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Biochemistry

Phorbol esters potentiate tyrosine phosphorylation of epidermal growth factor receptors in A431 membranes by a calcium-independent mechanism

(protein kinase C/mitogenesis/epidermal growth factor receptor affinity)

SOON OK MOON*, H. CLIVE PALFREY†, AND A. CHRISTIE KING*

*Department of Biological Chemistry, University of Illinois Health Science Center, Chicago, IL 60612; and †Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637

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ABSTRACT **Incubation of membranes prepared from A431 cells with either epidermal growth factor (EGF) or phorbol 12-myristate 13-acetate (PMA) stimulates the transfer of ^{32}phosphate from [γ-^{32}P]ATP into 8–10 membrane proteins. The major phosphorylated protein migrates on NaDodSO$_4$/polyacrylamide gels with an apparent M_r of 180,000, corresponding to the previously identified EGF receptor. Stimulation of EGF receptor phosphorylation by PMA does not require Ca^{2+}, suggesting that prior activation of protein kinase C is not a prerequisite for phosphate transfer. PMA-enhanced phosphorylation proceeds at 4°C and requires Mn^{2+}, both properties of tyrosine-specific protein kinases. Phospho amino acid analysis of the M_r 180,000 receptor band shows that only tyrosine residues are phosphorylated when A431 membranes are treated with either EGF or PMA. Moreover, proteolysis reveals that these residues are located in the same peptides of the receptor. These results demonstrate that a potent tumor-promoting phorbol ester can mimic a critical early response usually elicited by EGF.**

Phorbol ester tumor promoters are mitogenic and induce phenotypic changes in mammalian cells that resemble transformation by oncogenic viruses (1–3). The mechanisms that direct tumor promotion, transformation, and growth potentiation are not known; however, recent evidence indicates that protein kinases may be involved in mediation of these effects. Transformation by Rous sarcoma virus (RSV) requires the activity of a virally encoded tyrosine-specific protein kinase (4). The viral gene is apparently derived from a normal cellular gene and is present in transformed cells at a level 50- to 100-fold more abundant than in normal cells (5–8). Tyrosine-specific protein kinase activity is also stimulated after treatment of cells and membranes with several potent polypeptide mitogens, including epidermal growth factor (EGF), insulin, and platelet-derived growth factor (9–11). In several instances, the major substrate for mitogen-stimulated tyrosine phosphorylation is the receptor specific for that particular mitogen (10–14). For EGF and insulin receptors, the tyrosine-specific protein kinase activity is associated with affinity-purified forms of the receptor and is believed to be an integral part of the receptor itself (14, 15). The finding that different activators of cell growth enhance phosphorylation of tyrosine residues has led to the speculation that tyrosine-specific protein kinases may represent cellular control elements that regulate normal and pathologic growth.

Receptors for phorbol esters (16–18) are tightly associated with another protein kinase activity (19, 20), protein kinase C. Both phorbol binding (19–21) and kinase activity (22–24) are dependent on the simultaneous presence of Ca^{2+} and phospholipid. Protein kinase C is activated by diacylglycerol generated after hormonal stimulation of phosphatidyl inositol breakdown (23, 24). However, tumor-promoting phorbol esters can substitute for diacylglycerol in the activation of protein kinase C *in vitro* (20, 23, 25) and can lower the Ca^{2+} requirement for activation (23, 25).

Tumor promoters could affect cellular proliferation by either modulating the binding of mitogens to their specific receptors or altering the activity of receptor-associated protein kinases specific for tyrosine residues (or both). In normal chicken embryo fibroblasts, it was reported that phorbol esters alter gene expression and protein phosphorylation (26) and stimulate tyrosine phosphorylation of a M_r 42,000 protein (27). Phorbol ester tumor promoters also alter binding of EGF to a variety of different cell types through inhibition of a class of high-affinity receptors (28–30). The reduction of EGF receptor affinity correlates with the potency of tumor-promoting activity for phorbol analogs (1, 30, 31) and with the binding affinity to specific phorbol receptors (16–18). It is not presently known whether phosphorylation of EGF receptors is related to alterations in binding affinity. Phorbol esters may affect EGF receptor affinity in one of several ways. One possibility is that phorbol esters may directly alter receptor-mediated tyrosine phosphorylation reactions. Alternatively, phorbol esters may activate protein kinase C, which either indirectly modifies the activity of a tyrosine protein kinase or directly phosphorylates the EGF receptor.

In this report, we investigate the effects of phorbol 12-myristate 13-acetate (PMA) on phosphorylation of the EGF receptor and other substrates in membranes prepared from A431 cells. A431 cells have a high density of EGF receptors and have provided a very useful model system for study of the EGF-enhanced phosphorylation response (32–34). We find that phorbol esters, like EGF, stimulate tyrosine phosphorylation of the EGF receptor. This indicates that treatment of A431 cell membranes with PMA mimics a critical early response normally elicited by EGF. The phosphorylation proceeds in the absence of Ca^{2+}, suggesting that prior activation of protein kinase C is not required for phosphorylation of the EGF receptor.

METHODS

Cell Culture and EGF. A431 cells, provided by Graham Carpenter, were grown in Dulbecco's modified Eagle's medium containing Earle's balanced salts, 2 mM glutamine, streptomycin and penicillin each at 100 units/ml, and 10% fetal calf serum and were equilibrated with 5% CO_2/95% air. Mouse EGF was purified from adult male submaxillary

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Abbreviations: RSV, Rous sarcoma virus; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate.

glands (Pel-Freez) by the methods described by Savage and Cohen (35).

Preparation of Membranes. A431 cells were scraped free from culture dishes in harvest buffer (0.05 M borate/0.15 M NaCl/1 mM $MgCl_2$, pH 7.2), resuspended into 2 pellet vol of lysis buffer (0.02 M sodium borate/0.2 mM EDTA/2 mM phenylmethylsulfonyl fluoride, pH 10.2), stirred with a Vortex mixer, and pelleted at 25,000 × *g* for 30 min. Plasma membranes were then purified as described (36, 37).

EGF-Enhanced Phosphorylation Assay. The reaction mixture consisted of 20–50 μg of membrane protein incubated with 20 mM Hepes, pH 7.4/1 mM $MnCl_2$/15 μM [γ-^{32}P]ATP (≈10^3 cpm/pmol)/40 ng of EGF/7.5 μg of bovine serum albumin in a final volume of 60 μl. Membranes were preincubated on ice with or without EGF for 10 min and the reaction was initiated by the addition of labeled ATP. All incubations took place for 10 min in an ice bucket. These conditions give maximal EGF receptor phosphorylation in A431 membranes and inhibit phosphatase activity (32–34). The reaction was terminated by the addition of $NaDodSO_4$ sample buffer and boiling for 5 min.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Autoradiography. Phosphorylated membrane components were separated by $NaDodSO_4$ gel electrophoresis by using a 5–15% exponential polyacrylamide gradient slab gel (38). The molecular weight standards used to calibrate the gels were: spectrin, M_r 240,000 and 220,000; β-galactosidase, M_r 130,000; bovine serum albumin 68,000; carbonic anhydrase, M_r 29,000; hemoglobin, M_r 16,000. The gels were fixed and dried under vacuum. Autoradiography was performed with Kodak XAR-5 film.

Phospho Amino Acid Analysis and Tryptic Peptide Maps. The M_r ≈180,000 phosphoprotein was located in dried gels by means of an autoradiograph and was extracted from $NaDodSO_4$ gels by trypsin as described (39). For phospho amino acid analysis, the tryptic peptides were subjected to acid hydrolysis under vacuum for 2 hr and the resultant phospho amino acids were analyzed by two-dimensional thin-layer electrophoresis (first dimension: pH 1.9, 500 V for 2 hr; second dimension: pH 3.5, 500 V for 1 hr). Markers were detected with ninhydrin. For peptide maps, the trypsinized receptor phosphopeptides were separated on cellulose thin-layer plates by electrophoresis at pH 3.5 (anode at left) and ascending chromatography as described (39).

RESULTS

Phorbol Esters Enhance Phosphorylation of Identical Substrates as EGF in A431 Membranes. Membranes prepared from A431 cells were incubated with [γ-^{32}P]ATP at 4°C, and endogenous protein kinase activity was measured in the presence or absence of EGF and PMA. The membranes were prepared in the absence of Ca^{2+} to ensure that limited proteolysis of the receptor by a cytosolic, Ca^{2+}-dependent, neutral protease did not occur during cell lysis (40–43) and thereby reduce tyrosine-specific kinase activity associated with the EGF receptor (41, 42). All incubations were at 4°C, because phosphatases and protein kinases other than the EGF-receptor tyrosine-specific kinase are inhibited at this temperature in A431 membranes (14, 32, 33). The incubations contained both Mn^{2+} and Ca^{2+} ions: Mn^{2+} maximally stimulates tyrosine-specific kinase activity in these membranes (14, 32) and Ca^{2+} is required for protein kinase C activity (22–24). Incubation of membranes with 0.1 μM EGF at 4°C in the presence of Mn^{2+} and Ca^{2+} enhanced the phosphorylation of several proteins displayed on 5–15% gradient $NaDodSO_4$/polyacrylamide gels (Fig. 1 *Left*, lane B) when compared to untreated membranes (lane A). The major substrate phosphorylated under these conditions was a band that migrated on 5–15% gradient polyacrylamide gels with M_r ≈180,000. This protein has been previously identified as the EGF receptor by affinity labeling and immunoprecipitation (14, 32–34, 40–44). When these same membranes were incubated with 0.1 μM PMA (lane C), phosphorylation was stimulated to a similar extent as that observed with EGF, and again the major substrate was the M_r ≈180,000 band. Incubation of membranes with 0.1 μM 4-β-phorbol, a nonpromoting analog, did not result in significant enhancement of phosphorylation of the receptor band or any other substrate (Fig. 1 *Right*, lane C). Enhanced phosphorylation by PMA was apparent at concentrations as low as 1 nM, but maximal phosphorylation activity required concentrations of PMA in the range of 0.01 to 0.1 μM (not shown). These results suggest that a tumor-promoting phorbol ester can activate endogenous protein kinase activity in A431 membranes and stimulate phosphorylation of both the EGF receptor and other minor substrates in an identical manner to EGF itself.

Phosphorylation of A431 Membranes with PMA Is Ca^{2+} Independent. Because protein kinase C activity is a Ca^{2+}-dependent enzyme that is stimulated by phorbol tumor promoters, we studied whether phosphorylation of the EGF receptor could proceed in Ca^{2+}-free buffers containing 0.1 mM EGTA, a calcium-specific chelator. EGTA has been shown to prevent protein kinase C activity (28). All incubations were at 4°C and contained Mn^{2+}. Untreated membranes show a low level of endogenous kinase activity (Fig. 2, lane A). EGTA and Ca^{2+} do not alter the basal level of ^{32}phosphate incorporation under the assay conditions employed (not shown). Phosphorylation of the M_r ≈180,000 EGF receptor band is enhanced when membranes are incubated with PMA at 0.1 μM (lane C), PMA with 1 mM Ca^{2+} (lane D), or PMA with 0.1 mM EGTA (lane E). Under these incubation conditions, Ca^{2+} appears to be neither stimulatory nor inhibitory to the phosphorylation response. Phosphorylation is also not affected by EGTA. These results indicate that the major phosphorylated product in Ca^{2+}-free buffer is the EGF receptor and suggest that protein kinase C is not responsible for the phosphate transfer assayed here.

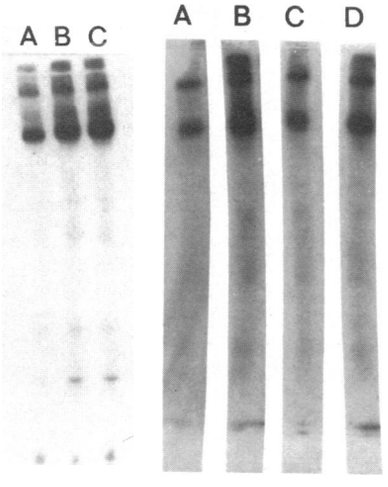

FIG. 1. (*Left*) Phosphorylation of A431 membranes with EGF or PMA. A431 membranes were prepared in EDTA-containing buffers to ensure that the EGF receptor was not proteolyzed by a Ca^{2+}-dependent cytosolic protease. The membranes were incubated under the conditions described (see text), except that 1 mM Ca^{2+} was present. The membranes were untreated (lane A) or were incubated with 0.1 μM EGF (lane B) or 0.1 μM PMA (lane C). (*Right*) Comparison of A431 membrane phosphorylation with EGF, PMA, or 4-β-phorbol. A431 membranes were incubated under identical conditions described in *Left*, except that Ca^{2+} was omitted from the incubation. The conditions are: untreated (lane A), 0.1 μM EGF (lane B), 0.1 μM 4-β-phorbol, a nonpromoting phorbol analog (lane C), and 0.1 μM PMA (lane D).

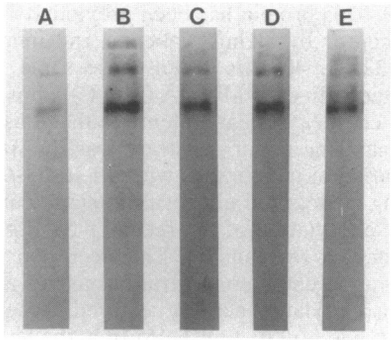

FIG. 2. Effect of Ca^{2+} and EGTA, a calcium-specific chelator, on phosphorylation in A431 membranes stimulated by PMA. A431 membranes were incubated with [γ-^{32}P]ATP in the presence of Mn^{2+} and the following additions made: lane A, untreated; lane B, 0.1 μM EGF; lane C, 0.1 μM PMA; lane D, 0.1 μM PMA with 1 mM Ca^{2+}; lane E, 0.1 μM PMA with 0.1 mM EGTA.

Interestingly, when Ca^{2+} and Mn^{2+} were present simultaneously with 0.1 μM EGF and 0.1 μM PMA, phosphorylation of the M_r ≈180,000 receptor band appeared to be additive (Fig. 3 *Left*, lanes B–D). Omission of Ca^{2+} and inclusion of 0.1 mM EGTA prevented this effect (Fig. 3 *Right*, compare lanes C and E with lanes D and F). On some autoradiographs, when Ca^{2+} was present, there was visible just slightly above the receptor band a unique and diffuse band with M_r ≈210,000. The appearance of this new Ca^{2+}-dependent phosphorylated substrate parallels the disappearance of radioactivity in both the highest molecular weight (M_r 300,000) and lowest molecular weight substrates observed on this gel.

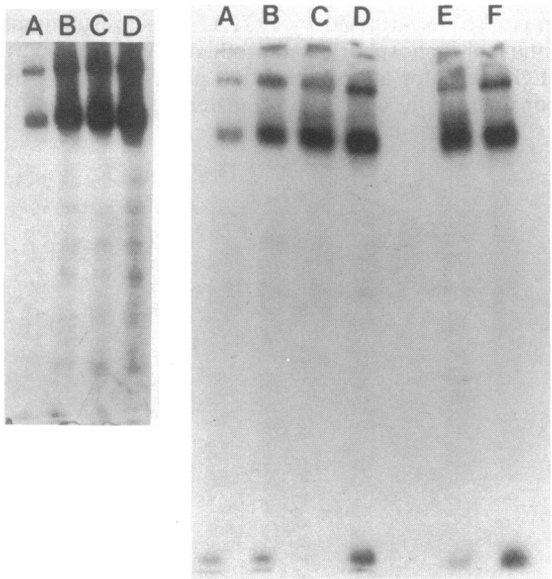

FIG. 3. Ca^{2+} potentiates phosphorylation response to PMA and EGF. (*Left*) A431 membranes were incubated in the presence of both 1 mM (each) Ca^{2+} and Mn^{2+}. The conditions are: lane A, untreated; lane B, 0.1 μM EGF; lane C, 0.1 μM PMA; lane D, 0.1 μM EGF and 0.1 μM PMA. (*Right*) A431 membranes were incubated as described (see text), except that in some samples 1 mM Ca^{2+} was present (lanes C and E) or 0.1 mM EGTA (lanes A, B, D, and F). The conditions are 0.1 mM EGTA (lane A), 0.1 μM EGF with 0.1 mM EGTA (lane B), 0.1 μM EGF, 0.1 μM PMA, and 1 mM Ca^{2+} (lanes C and E), or EGF, PMA, and 0.1 mM EGTA (lanes D and F). These results show that when EGF or both EGF and PMA are present, there is phosphorylation of the M_r 180,000 receptor band independent of the presence (lanes C and E) or absence (lanes D and F) of Ca^{2+}. When calcium is present (lanes C and E), the overall phosphorylation pattern is altered.

Data from two separate incubations are shown in Fig. 4. In both instances, the presence of Ca^{2+} results in the appearance of a phosphorylated substrate that runs slightly slower than the receptor band (lanes C and E). This phosphoprotein is not present in Ca^{2+}-free buffers containing low levels of EGTA (lanes D and F). These results show that when the phosphorylation reaction proceeds in the presence of Ca^{2+}, EGF, and PMA, there are at least three changes in the phosphorylation pattern not observed in Ca^{2+}-free conditions Phosphorylation of one unique band is enhanced, and two other bands are phosphorylated to a lesser extent.

When Mn^{2+} is removed from the incubation and Ca^{2+} and Mg^{2+} are the only divalent cations present, phosphorylation at 4°C is negligible in the presence of either EGF or PMA (Fig. 4). Transfer of ^{32}phosphate from ATP to a variety of A431 membrane substrates is enhanced in the presence of Mn^{2+} when incubated with either 0.1 μM EGF (lane B) or 0.1 μM PMA (lane C) when compared to untreated membranes (lane A). These results strengthen the speculation that a Ca^{2+}-dependent protein kinase is not responsible for the phosphorylation activity directed towards the EGF receptor in A431 membranes when PMA is present.

PMA and EGF Stimulate Phosphorylation of Tyrosine Residues in Identical Peptides of the EGF Receptor. The properties of the phosphorylation response to PMA assayed in A431 membranes suggest that a tyrosine-specific protein kinase is responsible. Phospho amino acid analysis of the M_r ≈180,000 EGF receptor showed that when phosphorylation is stimulated by either EGF (0.1 μM) or PMA (0.1 μM), the only residue phosphorylated is tyrosine (Fig. 5). Analysis of tryptic digests of the receptor revealed three major phosphopeptides that were identical after stimulation with either EGF or PMA (Fig. 6) and that were not present in untreated membranes (not shown). No new phosphorylated peptides were found when PMA was used as stimulant. These results show that in A431 membranes, PMA stimulates phosphorylation of the EGF receptor in an identical manner as EGF itself.

DISCUSSION

In this report, we show that the tumor promoter PMA, like EGF, stimulates phosphorylation in A431 membranes (Fig. 1). The phosphorylation reaction has the properties expected of the EGF receptor-associated tyrosine-specific protein kinase: it is Mn^{2+} dependent, proceeds at reduced temperatures, and occurs only on tyrosine residues (Figs. 1–5). The enhanced phosphorylation occurs at low concentrations of tumor-promoting phorbol analogs and does not occur when membranes are treated with nonpromoting analogs (Fig. 1 *Right*). These results indicate that a tumor-promoting phor-

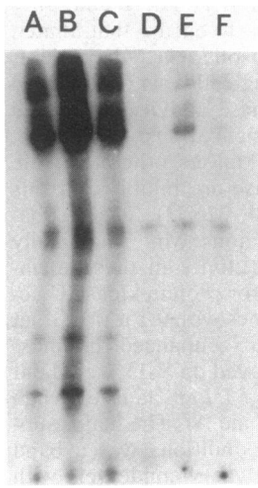

FIG. 4. Phosphorylation of A431 membranes is inefficient when Mn^{2+} is replaced by Ca^{2+}. A431 membranes were incubated with [γ-^{32}P]ATP either in the presence of Mn^{2+} (lanes A–C) or with 1 mM (each) Mg^{2+} and Ca^{2+} (lanes D–F). The membranes were then incubated at 4°C for 10 min under the following conditions: untreated (lanes A and D), 0.1 μM EGF (lanes B and E), or 0.1 μM PMA (lanes C and F).

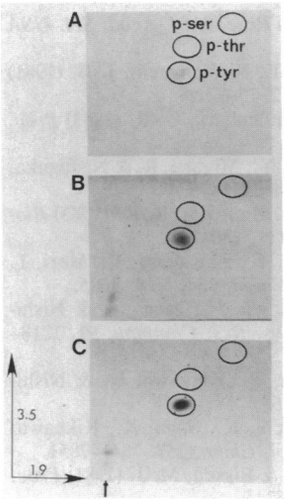

FIG. 5. Phospho amino acid analysis of the M_r ≈180,000 EGF receptor band. A431 membranes were untreated (*A*) or were incubated with [γ-^{32}P]ATP with either 0.1 μM EGF (*B*) or 0.1 μM PMA (*C*) for 10 min at 4°C. After solubilization in NaDodSO$_4$ sample buffer and electrophoresis, the M_r 180,000 receptor band was localized by autoradiography, excised from the gel, and extracted with trypsin. The peptides generated were then hydrolyzed under vacuum, and the phospho amino acids (phosphoserine, phosphotyrosine, and phosphothreonine) were separated by two-dimensional thin-layer electrophoresis.

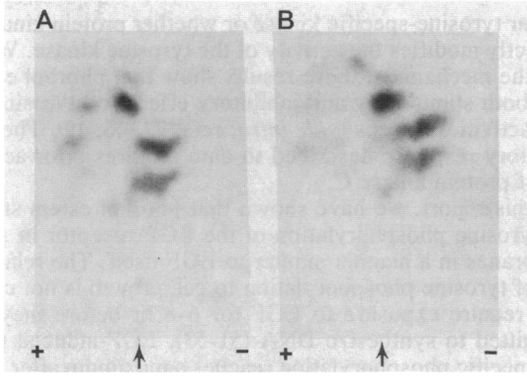

FIG. 6. Tryptic peptide maps of M_r 180,000 EGF receptor. A431 membranes were either treated with 0.1 μM EGF (*A*) or 0.1 μM PMA (*B*) and incubated with [γ-^{32}P]ATP for 10 min at 4°C. The EGF phosphorylated receptor was localized on dried gels and extracted from NaDodSO$_4$ gel slices as described in the legend to Fig. 4. Phosphopeptides were separated on cellulose thin-layer plates by electrophoresis at pH 8.9 (anode at left) and ascending chromatography (39).

bol analog is capable of mimicking an *in vitro* response elicited by the mitogen EGF: tyrosine phosphorylation of the EGF receptor.

Recent evidence has implicated the activation of protein kinase C by phorbol esters as a possible link to tumor promotion (19–25). This suggests that one of the early effects of treating cells with phorbol esters is to direct the Ca^{2+}- and phospholipid-dependent phosphorylation of specific cellular substrates. The PMA-stimulated phosphorylation found in the present work is unlikely to be due to protein kinase C. Phosphorylation occurs in membrane preparations largely stripped of peripheral membrane proteins by lysis in pH 10.2 buffers contalning EDTA. Protein kinase C would not be expected to remain associated with the membrane under these conditions (19). In support of this concept, we are unable to assay protein kinase C activity in A431 membranes using exogenous substrates (not shown). In addition, phosphorylation is independent of the presence of Ca^{2+}. Finally, phosphorylation is directed exclusively towards tyrosine residues. Protein kinase C-directed phosphorylation occurs at serine or threonine residues (or both) and never on tyrosine residues. These results make it unlikely that residual membrane-associated protein kinase C activity is responsible for phosphorylation of the EGF receptor, either directly or indirectly.

Recent evidence suggests that there may exist multiple types of phorbol receptors, a cytoplasmic binding component that requires Ca^{2+} and phospholipid and a membrane component that is Ca^{2+} and phospholipid independent (45). We speculate that the enhanced phosphorylation that we assayed in A431 membranes is mediated by a membrane, Ca^{2+}-independent phorbol receptor rather than protein kinase C. An alternate hypothesis is that PMA binds directly to the EGF–receptor–kinase complex, but there are no kinetic binding data available to support this contention. Until now, protein kinase C was the only enzyme known to be affected by phorbol tumor-promoting agents. Our data suggest that PMA is also capable of stimulating another protein kinase: the tyrosine-specific protein kinase associated with the EGF receptor.

An immediate effect of treatment of intact cells with phorbol esters is a reduction in the apparent affinity of EGF receptors (28–30). This effect, like activation of protein kinase C, correlates with the potency of phorbol analogs for tumor promotion and binding of phorbol esters to their specific receptors. These results have suggested that protein kinase C-directed phosphorylation of the EGF receptor or an unidentified regulatory factor is responsible for the phorbol ester-induced reduction in EGF receptor affinity. In this report, we have shown that PMA stimulates tyrosine phosphorylation of the EGF receptor. The accumulated evidence indicates that tyrosine phosphorylation is not responsible for the phorbol ester-induced affinity changes in the EGF receptor. Phorbol esters do not cause a reduced EGF receptor affinity when added directly to membranes or when intact cells are incubated at reduced temperatures (28, 29). The enhanced receptor phosphorylation assayed here occurs in A431 membrane preparations and takes place at 4°C. This indicates that receptor phosphorylation under these conditions is not likely to be responsible for EGF receptor affinity changes. In addition, the PMA-stimulated phosphorylation of A431 membranes is largely independent of the presence of Ca^{2+}. We have found that Ca^{2+} is a critical regulator of EGF receptor affinity (unpublished data). These results implicate a regulatory function for a Ca^{2+}-dependent enzyme in mediation of altered affinity states of the EGF receptor.

We find that PMA stimulates tyrosine phosphorylation of the EGF receptor at 4°C in membranes prepared from A431 cells that lack protein kinase C. When these same membranes are reconstituted with purified protein kinase C, stimulation with phorbol esters causes receptor phosphorylation at threonine residues (46). The result of protein kinase C-induced phosphorylation of the receptor is a reduction in the endogenous tyrosine-specific kinase activity (ref. 46; unpublished results). Thus, our ability to observe enhanced EGF receptor phosphorylation at tyrosine residues in A431 membranes is probably dependent on freedom from contamination by protein kinase C enzyme activity.

Unlike the situation in isolated A431 membranes, the major portion of receptor phosphorylation in intact cells stimulated with EGF is directed towards serine and threonine residues, and only a minor portion is found as phosphotyrosine (9, 44, 46). These results suggest that protein kinase C may be responsible in part for phosphorylation of the EGF receptor, *in vivo*, and that autophosphorylation at tyrosine is only a minor component of the overall phosphate incorporation in intact metabolically active cells. Vinculin (47) and insulin receptors (48) are other substrates phosphorylated by both protein kinase C and tyrosine-specific kinases.

Phorbol esters and diacylglycerol also stimulate tyrosine phosphorylation of a cytosolic substrate of M_r 42,000 in intact cells (49). Rapid tyrosine phosphorylation of this substrate appears to be ubiquitous after stimulation with a variety of mitogenic substances (50). At present, it is unclear whether this response is caused directly by stimulation of a

cellular tyrosine-specific kinase or whether protein kinase C indirectly modifies the activity of the tyrosine kinase. Whatever the mechanism, these results show that phorbol esters have both stimulatory and inhibitory effects on tyrosine kinase activities in cells (*vide infra*; refs. 27, 46, 49). The one inhibitory response described to date requires prior activation of protein kinase C.

In this report, we have shown that phorbol esters stimulate tyrosine phosphorylation of the EGF receptor in A431 membranes in a manner similar to EGF itself. The relationship of tyrosine phosphorylation to cell growth is not clear. Cells require exposure to EGF for 6–8 hr before they are committed to synthesize DNA (51–53). EGF-induced tyrosine-specific phosphorylation reaches a maximum after 5–10 min (10, 32, 33). Thus, there is no temporal correlation between EGF-enhanced tyrosine phosphorylation and the growth response. In addition, an analog of EGF, cyanogen bromide-cleaved EGF, is reported to be capable of enhancing receptor phosphorylation but is not mitogenic (54), and EGF does not elicit a mitogenic response in A431 cells at concentrations that stimulate EGF-dependent tyrosine protein kinase activity in membranes (55). More recent results show that cultures of senescent fibroblasts that do not respond to EGF have nondetectable levels of EGF-receptor tyrosine-specific kinase activity, whereas responsive cultures express this same kinase activity (56). These studies indicate that receptor phosphorylation may be a necessary but insufficient signal to induce a growth response. Our results suggest that phorbol esters may initiate a biochemical cascade leading to a mitogenic response because of their ability to mimic an early response known to be elicited by EGF, enhanced tyrosine phosphorylation of EGF receptors.

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