Inhibition of the apparent affinity of the epidermal growth factor receptor caused by phorbol diesters correlates with phosphorylation of threonine-654 but not other sites on the receptor

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Addition of 4 β -phorbol 12 β -myristate 13 α -acetate (PMA) to A431 human epidermoid carcinoma cells causes a marked increase in the phosphorylation state of the epidermal growth factor (EGF) receptor with a concomitant inhibition of both the high-affinity binding of ¹²⁵I-EGF and the receptor tyrosine kinase activity. It was found in the present studies that the diuretic drug amiloride has no effect on the action of PMA to inhibit the binding of ¹²⁵I-EGF. However, amiloride was observed to inhibit markedly the effect of PMA to cause a 3-fold increase in the phosphorylation state of the EGF receptors. In the presence of PMA and amiloride, the increase in the phosphorylation state of the EGF receptors was found to be only 1.2-fold over controls. Analysis of the EGF receptor phosphorylation sites by phosphopeptide mapping by reverse-phase h.p.l.c. demonstrated that PMA increases the phosphorylation state of the EGF receptor at many sites. One of these sites has been identified as a C-kinase substrate, threonine-654. In the presence of amiloride, PMA causes phosphorylation of threonine-654 to the same stoichiometry as that observed in the absence of amiloride. However, the marked increase in the phosphorylation state of the EGF receptor at other sites caused by PMA is abolished in the presence of amiloride. We conclude that the extensive phosphorylation of the EGF receptor at several sites caused by the addition of PMA to A431 cells is not required for the action of PMA to inhibit the high-affinity binding of ¹²⁵I-EGF. The results indicate that the phosphorylation state of threonine-654 may play a role in this process.

INTRODUCTION

Tumour-promoting phorbol diesters such as PMA cause the phosphorylation of the EGF receptor on serine and threonine residues when added to intact A431 cells (Cochet *et al.*, 1984; Iwashita & Fox, 1984; Davis & Czech, 1984). Most of the increase in EGF receptor phosphorylation due to PMA occurs at sites which are also phosphorylated on the EGF receptor isolated from control cells (Iwashita & Fox, 1984; Davis & Czech, 1985a). A part of the increase in the level of [³²P]phosphate associated with the EGF receptor is at a unique site which is not phosphorylated on the EGF receptors of control cells (Cochet *et al.*, 1984; Iwashita & Fox, 1984; Davis & Czech, 1985a). This site has been identified as threonine-654, which is located 9 residues distant from the transmembrane domain of the EGF receptor on the cytoplasmic side of the plasma membrane (Hunter *et al.*, 1984; Davis & Czech, 1985a).

Associated with this increase in the phosphorylation state of the EGF receptor is an inhibition of the high-affinity binding of ¹²⁵I-EGF to cultured cells (Brown *et al.*, 1979; Lee & Weinstein, 1979; Shoyab *et al.*, 1979; Davis & Czech, 1984) and an inhibition of the EGF-stimulated tyrosine kinase activity of the EGF receptor (Cochet *et al.*, 1984; Friedmann *et al.*, 1984;

Davis *et al.*, 1985). It has been suggested that the increase in the phosphorylation state of the EGF receptor caused by phorbol diesters may be mechanistically related to the effects of phorbol diesters on the function of the EGF receptor. A key problem raised by this hypothesis is the resolution of the functional roles of the different phosphorylation sites on the EGF receptor that are increased when cells are treated with PMA. Which of these sites, if any, are causally related to the response of cultured cells to phorbol diesters?

In order to address this question, we have made use of the diuretic drug amiloride, which acts as a protein kinase inhibitor (Ralph *et al.*, 1982; Holland *et al.*, 1983; Davis & Czech, 1985b) that is competitive with ATP. Addition of this drug to the EGF receptor *in vitro* causes an inhibition of the EGF-stimulated tyrosine kinase activity of the receptor (Davis & Czech, 1985b). In intact A431 cells, amiloride decreases the phosphorylation state of the EGF receptor (Davis & Czech, 1985b). We now report that amiloride has no effect on the ability of PMA to cause inhibition of the high-affinity binding of ¹²⁵I-EGF to A431 cells. However, under these conditions, the effect of PMA to increase the phosphorylation state of the EGF receptor is markedly reduced. We conclude that the extensive phosphorylation of the EGF receptor at many sites that is observed when cells are treated with PMA is

Abbreviations used: EGF, epidermal growth factor; PMA, 4 β -phorbol 12 β -myristate 13 α -acetate; amiloride, *N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide; NaDodSO₄, sodium dodecyl sulphate; TPCK-treated trypsin, tosylphenylalanylchloromethane-treated trypsin.

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not required for the action of PMA to inhibit the apparent affinity of the EGF receptor. Receptor modulation occurs under conditions where only one major phosphorylation site, threonine-654, is phosphorylated in response to PMA when amiloride is present.

EXPERIMENTAL PROCEDURES

Materials

EGF was prepared (Savage & Cohen, 1972; Matrisian *et al.*, 1982) and iodinated (Pessin *et al.*, 1983) as described. [32 P]Phosphate was from New England Nuclear. TPCK-treated trypsin was obtained from Worthington. Trifluoroacetic acid and triethylamine were from Pierce. Acetonitrile was from Burdick and Jackson. PMA was obtained from Sigma. Amiloride was a gift from Dr. C. Stone (Merck, Sharp and Dohme).

Methods

Cell culture. A431 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum.

Binding of 125 I-EGF to A431 cell monolayers. A431 cells were seeded in 16 mm wells and grown to a density of 2×10^4 cells/well. The cells were then washed and incubated in 0.5 ml of 120 mM-NaCl/6 mM-KCl/1.2 mM-MgSO₄/1 mM-CaCl₂/25 mM-Hepes (pH 7.4)/10 mM-glucose. The cells were incubated with and without amiloride for 2 h at 37 °C and then treated with and without 10 nM-PMA for 30 min. The cells were then cooled to 0 °C and 100 pM- 125 I-EGF was added. After 4 h the cells were washed four times with cold medium and then solubilized with 0.5 ml of 0.4 M-NaOH. Radioactivity was quantified with a gamma counter. Non-specific binding was determined in parallel incubations in the presence of a 1000-fold excess of EGF.

Analysis of the phosphorylation state of the EGF receptor. A431 cells were seeded in 35 mm wells and grown to a density of 2×10^5 cells/well. The monolayers were then washed and incubated with 1 ml of phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 0.5 mCi of [32 P]phosphate/ml. To achieve isotopic equilibrium, the cells were incubated for 24 h. The cells were then treated with and without 10 nM-PMA for 30 min. The monolayers were washed once and the cells were lysed with 1.5 ml of 1.5% Triton X-100/1% sodium deoxycholate/0.1% NaDodSO₄/0.5 M-NaCl/5 mM-EDTA/50 mM-NaF/100 μ M-Na₃VO₅/1 mM-phenylmethanesulphonyl fluoride/10 μ g of leupeptin $\cdot$ ml⁻¹/25 mM-Hepes (pH 7.8). The lysate was clarified by centrifugation at 4 °C for 30 min at 100000 g. A 1 ml sample of the supernatant was then mixed with 10 μ l of a 1:10 dilution of anti-(EGF receptor) serum. This antiserum was obtained from a rabbit which had been injected with an affinity-purified preparation of A431 cell EGF receptor. After 60 min of incubation at 22 °C on an end-over-end mixer, 20 μ l of protein A-Sepharose CL-4B (Pharmacia) was added and the incubation was continued for a further 60 min. The immunoprecipitate was collected by centrifugation, extensively washed with lysis buffer and finally washed with 0.1% NaDodSO₄/0.2% Triton X-100/25 mM-Hepes (pH 7.8).

Phosphopeptide mapping. Immunoprecipitated EGF receptor in a volume of 100 μ l was reduced by the addition of 80 μ l of 10% NaDodSO₄/14 mM-dithiothreitol and heated at 60 °C for 15 min. The EGF receptor was then alkylated with 40 μ l of 0.4 M-iodoacetamide dissolved in 0.25 M-Tris/HCl (pH 8.6). After 15 min of incubation at room temperature, 100 μ l of 2-mercaptoethanol/glycerol (1:3, by vol.) was added and the samples were heated at 60 °C for 15 min. The reduced and alkylated EGF receptor was then resolved from other proteins by electrophoresis on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO₄. The EGF receptor in the unfixed gel was located by autoradiography at 4 °C for 60 min and excised from the gel. The gel slice was extensively washed with water and then equilibrated with 50 mM-NH₄HCO₃. The gel slice was resuspended in 3 ml of 50 mM-NH₄HCO₃ and incubated at 37 °C with 50 μ g of TPCK-treated trypsin/ml. After 5 h a second addition of trypsin was made and the incubation was continued for a further 19 h.

Phosphopeptides released from the gel slice were resolved by reverse phase h.p.l.c. The chromatography apparatus consisted of a U6K injector, 6000 and M-4 solvent delivery systems, a model 450 variable wavelength detector, a model 440 fixed wavelength detector, a model 720 system controller, and a data module, all from Waters. The phosphopeptides were injected into a Waters μ Bondapak C-18 column (0.39 cm $\times$ 30 cm) equilibrated with 0.1% trifluoroacetic acid/0.05% triethylamine. The column was washed for 5 min and phosphopeptides were eluted with a linear gradient of acetonitrile in the above solvent over 45 min. The flow rate was 1 ml/min and fractions were collected at 30 s intervals. [32 P]Phosphopeptides were detected by measuring the Čerenkov radiation associated with each fraction in a β -counter.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed by the method of Hunter & Sefton (1980) as described (Davis & Czech, 1984).

RESULTS

Amiloride has been characterized as an inhibitor of Na⁺/H⁺ exchange across the plasma membrane of many cultured cell lines (Schuldiner & Rozengurt, 1982; Moolenaar *et al.*, 1982; Pouyssegur *et al.*, 1982; Frelin *et al.*, 1983) including A431 cells (Rothenburg *et al.*, 1983). Recently, PMA has been shown to stimulate an amiloride-sensitive Na⁺ uptake by HL60 cells (Besterman & Cuatrecasas, 1984). Rosoff *et al.* (1984) have suggested that the stimulation of an amiloride-sensitive Na⁺/H⁺ antiporter by PMA is an important signal for the differentiation of pre-B lymphocytes. However, Fig. 1 shows that amiloride does not block the effect of PMA in A431 cells to inhibit the high-affinity binding of 125 I-EGF. Amiloride failed to modulate PMA action at concentrations as high as 2 mM (Fig. 1), which are known to block Na⁺/H⁺ exchange. We conclude that a stimulation of Na⁺/H⁺ exchange is not required for the decrease in the apparent affinity of the EGF receptor caused by PMA. In addition, amiloride was observed to have no effect on the apparent affinity of the EGF receptor of control cells (Fig. 1).

It has been reported that amiloride is a potent inhibitor of protein kinases (Ralph *et al.*, 1982; Holland *et al.*, 1983; Davis & Czech, 1985b). We have observed that

Fig. 1. Effect of amiloride on the response of A431 cells to PMA

A431 cells were incubated at 37 °C with different concentrations of amiloride for 2 h. Subsequently, the cells were treated for 30 min with 10 nM-PMA. The cells were then cooled to 0 °C and 100 pM-¹²⁵I-EGF was added. After 4 h of incubation, the cell monolayers were washed four times with cold medium and solubilized with 0.5 ml of 0.4 M-NaOH. The radioactivity associated with the cells was measured with a gamma counter. Non-specific binding of ¹²⁵I-EGF was estimated in incubations containing 100 nM-EGF.

amiloride will inhibit the tyrosine kinase activity of the receptors for EGF, platelet-derived growth factor and insulin (Davis & Czech, 1985b). Amiloride added to intact A431 cells decreases the phosphorylation state of the EGF receptor in the absence and presence of EGF (Davis & Czech, 1985b). When cells were treated with PMA it was found that amiloride prevents most of the increase in the phosphorylation state of the EGF receptor caused by PMA (Fig. 2). As amiloride has no effect on the action of PMA to inhibit the high-affinity binding of ¹²⁵I-EGF to A431 cells (Fig. 1), this result appears to demonstrate that the effects of PMA on the apparent affinity of the EGF receptor can be resolved from its effects to cause receptor phosphorylation. The importance of this conclusion led us to investigate further the effect of amiloride on the phosphorylation state of the EGF receptor.

Phosphoamino acid analysis of the EGF receptor isolated from A431 cells demonstrated the presence of phosphoserine and phosphothreonine (Table 1). PMA treatment of the cells resulted in an increase in the level of both of these phosphoamino acids in the isolated EGF receptor. Addition of EGF to the cells caused the appearance of phosphotyrosine as well as an increase in the levels of phosphoserine and phosphothreonine. However, when the cells were first treated with amiloride, the effects of PMA and EGF on the phosphorylation state of the EGF receptor were altered (Table 1). Amiloride caused a marked reduction in the effect of PMA and EGF to increase the level of EGF receptor phosphoserine. Similarly a reduction in the level of phosphothreonine was also observed, but this effect was not as great as the

Fig. 2. Effect of amiloride on the phosphorylation state of the EGF receptor in intact A431 cells

A431 cells were labelled with [³²P]phosphate for 24 h. The cells were then treated with and without 1 mM-amiloride for 2 h. Subsequently, the cells were treated with and without 10 nM-PMA and 10 nM-EGF for 30 min. The cells were solubilized and the EGF receptors were immunoprecipitated and electrophoresed on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO₄. The Figure presents an autoradiogram of the dried gel. The autoradiogram is a 20 h exposure of Kodak X-OMAT AR film in the presence of Dupont Cronex Lightning Plus enhancing screen at -70 °C. The phosphorylation of the EGF receptor was quantified by densitometry of the autoradiograph. EGF and PMA were observed to cause a 4- and 3-fold increase in the phosphorylation state of the EGF receptor respectively. However, in the presence of amiloride, increases of only 1.8-fold and 1.2-fold were caused by EGF and PMA treatment respectively. Amiloride treatment alone decreased the phosphorylation state of the EGF receptor to half of that observed in control cells.

inhibition of the phosphorylation of serine residues (Table 1). Significantly, the [³²P]phosphoserine and [³²P]phosphotyrosine contents of the EGF receptors were actually lower in cells treated with PMA plus amiloride compared with control cells (no treatment). This result suggested that the effect of amiloride to decrease the phosphorylation state of the EGF receptor may be site-specific. This hypothesis was investigated by phosphopeptide mapping of the EGF receptor.

EGF receptors were isolated from [³²P]phosphate-labelled A431 cells and were digested with trypsin. The [³²P]phosphopeptides obtained were resolved by reverse-phase h.p.l.c. EGF increased the level of many phosphopeptides (Figs. 3a and 3c) which were found to contain phosphoserine and phosphothreonine. In addition, EGF caused a marked increase in the level of three phosphopeptides which were found to contain phospho-

Table 1. Phosphoamino acid analysis of the EGF receptor isolated from cells treated with and without amiloride

A431 cells were labelled with [32 P]phosphate for 24 h. The cells were then treated with and without 1 mM-amiloride for 2 h. Subsequently, the cells were treated with and without 10 nM-PMA or 10 nM-EGF for 30 min. The cells were solubilized and the EGF receptors were immunoprecipitated and electrophoresed on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO₄. The EGF receptors were located in the dried gel by autoradiography, excised from the gel, extracted, precipitated with trichloroacetic acid, partially hydrolysed with 6 M-HCl for 1 h at 110 °C and electrophoresed at pH 3.5 on 100 μ m cellulose-coated thin layer sheets. Phosphoamino acids were located by staining the plate with ninhydrin and were excised from the plate. The radioactivity associated with each phosphoamino acid was measured by Čerenkov counting in a β -counter. The results are expressed relative to the sum of the radioactivity measured in the phosphoamino acids derived from the EGF receptor of control cells (100%, 5321 c.p.m.).

Condition	Amiloride	Phosphoserine (%)	Phosphothreonine (%)	Phosphotyrosine (%)
Control	—	70.1	29.5	0.3
Control	+	28.2	19.3	< 0.1
EGF	—	180	78.4	18.1
EGF	+	115	52.1	6.9
PMA	—	175	92.0	0.3
PMA	+	53	81.4	< 0.1

tyrosine. Recently, these three sites of tyrosine phosphorylation on the EGF receptor have been identified as tyrosine-1068, tyrosine-1148 and tyrosine-1173 by Downward *et al.* (1984). One of these sites (tyrosine-1173) is a major site of phosphorylation on the EGF receptor in cells that have been treated with EGF (Fig. 3c). However, the other two sites on the EGF receptor are minor sites of phosphorylation (Fig. 3c). Amiloride partially inhibited the increase in the phosphorylation state of all the phosphopeptides caused by EGF (Fig. 3c).

In contrast to EGF, PMA did not stimulate the tyrosine phosphorylation of the EGF receptor, but was found to increase the phosphorylation state of all the phosphopeptides present in the EGF receptor isolated from control cells (Fig. 3b). In addition, PMA caused the appearance of a unique phosphopeptide which was eluted from the reverse-phase h.p.l.c. column at a very low acetonitrile concentration (Fig. 3b). Phosphoamino acid analysis of this phosphopeptide indicated the presence of [32 P]phosphothreonine. This phosphopeptide has been identified as Lys-Arg-Thr(P)-Leu-Arg (Davis & Czech, 1985a; Hunter *et al.*, 1984). The phosphorylated residue corresponds to threonine-654 of the EGF receptor (Davis & Czech, 1985a; Hunter *et al.*, 1984). Cochet *et al.* (1984) have estimated that the stoichiometry of phosphorylation of the EGF receptor at threonine-654 in A431 cells treated with PMA is 40%. Amiloride markedly reduced the level of all the phosphopeptides derived from the EGF receptor of PMA-treated cells, except the unique phosphopeptide Lys-Arg-Thr(P)-Leu-Arg that contains threonine-654 (Fig. 3b). Thus, the effect of amiloride to reduce the phosphorylation state of the EGF receptor in the presence of PMA is site-specific.

A comparison of the phosphopeptide maps of the EGF receptor isolated from control cells and from cells treated with PMA in the presence of amiloride is presented in Fig. 4. Under these conditions, PMA increases the phosphorylation state of only two phosphopeptides (Fig. 4). The major increase occurs on a phosphopeptide that contains the phosphorylation site threonine-654. A very small increase in the phosphorylation state of a peptide that was eluted from the reverse-phase column at 33 min was also observed. This phosphopeptide is the major phosphoryl-

ation site on the EGF receptor isolated from control cells (Fig. 3). Phosphoamino acid analysis indicated the presence of [32 P]phosphothreonine. In addition, the phosphorylation state of many other phosphopeptides was decreased on the EGF receptor isolated from cells treated with PMA and amiloride compared with control cells. This is in contrast to the effect of PMA in the absence of amiloride where the phosphorylation state of many phosphopeptides is observed to be increased (Fig. 3; Iwashita & Fox, 1984; Davis & Czech, 1985a). The effect of amiloride to block the phosphorylation of most of the phosphopeptides in the presence of PMA demonstrates that the action of PMA to inhibit the high-affinity 125 I-EGF binding to the EGF receptors of A431 cells does not require the extensive phosphorylation of the EGF receptors caused by PMA in the absence of amiloride.

DISCUSSION

Addition of PMA to intact A431 cells causes an increase in the phosphorylation state of the EGF receptor. In the absence of amiloride, an increase in the phosphorylation state of many sites on the EGF receptor is observed after the addition of PMA to A431 cells. However, in the presence of amiloride, PMA markedly increases the phosphorylation state of only one site when compared with the phosphorylation state of control cells (Fig. 4). This site has been identified as threonine-654 (Davis & Czech, 1985a; Hunter *et al.*, 1984). Amiloride was observed to have no direct effect on the action of PMA to inhibit the high-affinity binding of 125 I-EGF to A431 cells (Fig. 1). It is therefore suggested that the majority of the increase in the phosphorylation state of the EGF receptor is not causally related to the effect of PMA to decrease the apparent affinity of the EGF receptor.

The mechanism of the effect of PMA to inhibit the high-affinity binding of 125 I-EGF to A431 cells is not understood. The inhibition could be accounted for by either a reduction in the affinity of the EGF receptor, a decrease in the number of high-affinity binding sites, or by both of these mechanisms. As the high-affinity

Fig. 3. Phosphopeptide mapping of the EGF receptor isolated from cells treated with and without amiloride

A431 cells were labelled with [32 P]phosphate for 24 h and then treated with (----) and without (—) 1 mM-amiloride for 2 h. Subsequently, the cells were treated with and without 10 nM-PMA or 10 nM-EGF for 30 min. The cells were solubilized and the EGF receptors were immunoprecipitated, reduced and alkylated, electrophoresed on a 7% polyacrylamide gel and digested with trypsin. The [32 P]phosphopeptides obtained were resolved by reverse-phase h.p.l.c. on a Waters μ Bondapak C-18 column (0.39 cm $\times$ 30 cm) which had been equilibrated with 0.1% trifluoroacetic acid and 0.05% triethylamine. The column was washed for 5 min and the [32 P]phosphopeptides were eluted with a linear gradient of 0–45% (v/v) acetonitrile over 45 min. The flow rate was 1 ml/min. Fractions were collected every 30 s. The [32 P]phosphopeptides were detected by measuring the Čerenkov radiation with a β -counter. Phosphoamino acid analysis was performed on

Fig. 4. Effect of PMA in the presence of amiloride on the phosphopeptide map of the EGF receptor

The h.p.l.c. elution profiles of phosphopeptides derived from the EGF receptor of control cells and cells treated with PMA in the presence of amiloride were compared. The difference in the elution profiles is presented. The elution profile of the EGF receptor phosphopeptides isolated from control cells (no treatment, Fig. 3a) was subtracted from the profile obtained with the receptor isolated from cells treated with amiloride and PMA (Fig. 3b).

receptor population in A431 cells represents only about 10% of the total 125 I-EGF binding by the cells (Davis & Czech, 1984), the interpretation of binding isotherms to resolve these possibilities is not practical. Recent reports have indicated that the high-affinity 125 I-EGF binding sites of A431 cells can be distinguished from the low-affinity sites and therefore may represent a separate receptor species. First, monoclonal antibodies have been described that preferentially interact with one of the two classes of receptors (Kawamoto *et al.*, 1983; Gregoriou & Rees, 1984). Secondly, it has been demonstrated by the technique of fluorescence photobleaching recovery that the high-affinity receptors have a relatively restricted lateral mobility in the membrane, whereas the low-affinity receptors have a high degree of lateral mobility (Rees *et al.*, 1984). The effect of PMA may therefore be to reduce the number of high-affinity binding sites or to reduce the affinity of the high-affinity binding sites.

The results presented in the present paper indicate that extensive phosphorylation of the total pool of EGF receptors in A431 cells is not required for PMA action on the apparent affinity of the EGF receptor. However, if a low number of distinct high-affinity EGF receptors exist in A431 cells, it is possible that the stoichiometric phosphorylation of this class of receptors occurs under conditions where the phosphorylation stoichiometry of the total EGF receptor pool is extremely low. Thus, it is not possible to conclude that particular phosphorylation sites on the EGF receptor do not play a role in the regulation of the apparent affinity of the EGF receptor

each peak fraction by partial acid hydrolysis and thin layer electrophoresis. The results of this analysis are indicated on the chromatogram. The symbols S, T and Y represent phosphoserine, phosphothreonine, and phosphotyrosine. In each case, the principal phosphoamino acid detected is presented. Small amounts of other phosphoamino acids were found in several peaks and these are indicated in some cases in parentheses.

by PMA, even though the phosphorylation stoichiometry in the total population of EGF receptors at these sites is extremely low. However, the marked effect of PMA to cause phosphorylation of the EGF receptor at threonine-654 in the presence of amiloride (Fig. 3) suggests that there is a good correlation between the inhibition of high-affinity ^{125}I -EGF binding to A431 cells and the effect of PMA to cause phosphorylation of the EGF receptor at threonine-654. The position of this phosphorylation site on the EGF receptor is such that it could alter the function of the receptor. Threonine-654 is located 9 residues distant from the transmembrane domain of the EGF receptor on the cytoplasmic side of the plasma membrane in an extremely basic sequence:

Arg-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr(P)-
Leu-Arg-Arg.

This region of the receptor serves as a link between the EGF binding domain and the protein kinase domain (Ullrich *et al.*, 1984). If the protein kinase activity is regulated by allosteric conformational changes caused by the binding of EGF to the binding domain, this sequence would be of very great importance to the signal transmission. Perturbation of this conformational change because of phosphorylation of the EGF receptor at threonine-654 could affect the affinity of the EGF receptor. The very basic nature of the sequence surrounding threonine-654 suggests that it may interact electrostatically with anions on the surface of the plasma membrane. Possibilities include phospholipids and proteins associated with the membrane. Phosphorylation of threonine-654 and the introduction of an acidic residue into this very basic region of the EGF receptor could affect the interaction of the receptor with other cellular components. This could alter the affinity of the receptor or could signal the internalization and sequestration of the receptor away from the cell surface.

The EGF receptor can be phosphorylated *in vitro* at threonine-654 by C-kinase (Hunter *et al.*, 1984; Davis & Czech, 1985a). However, this phosphorylation *in vitro* of the EGF receptor does not alter the high-affinity binding of ^{125}I -EGF to A431 membranes (R. J. Davis & M. P. Czech, unpublished work). This result has also recently been reported by Fearn & King (1985) using KB cell membranes. These authors report that under conditions where C-kinase is maximally active (1 mM- Ca^{2+} in the presence of membranes) there is no effect of EGF receptor phosphorylation by C-kinase on the apparent affinity of the EGF receptor. However, in addition to this observation, Fearn & King (1985) report that there is a specific effect of PMA to inhibit ^{125}I -EGF binding to KB membranes, that requires C-kinase and is associated with tyrosine phosphorylation of the EGF receptor. Phosphorylation of threonine-654 was not observed by Fearn & King (1985) under these conditions. The relevance of this observation to intact cells is not clear. These results suggest that phosphorylation of the EGF receptor at threonine-654 does not directly cause an inhibition of ^{125}I -EGF binding. The failure of this *in vitro* system to demonstrate the inhibition of ^{125}I -EGF binding after the phosphorylation of the receptor at threonine-654 may be due to two reasons. (a) If the inhibition of binding observed in intact cells is due to the internalization of the high affinity receptors, the inhibition of binding of ^{125}I -EGF would not be observed in an experiment *in vitro*. Strong evidence to support this proposal has

recently been reported by Beguinot *et al.* (1985). (b) It is possible that phosphorylation of the EGF receptor at threonine-654 is necessary, but not sufficient, for the inhibition of ^{125}I -EGF binding to be observed and that some other modification of the receptor is required.

The ability of the diuretic drug amiloride to alter selectively the phosphorylation state of different sites on the EGF receptor is not understood. Amiloride has been shown to be a protein kinase inhibitor (Ralph *et al.*, 1982; Holland *et al.*, 1983; Davis & Czech, 1985b) which acts competitively with ATP. Addition of amiloride to a purified preparation of EGF receptors isolated from A431 membranes inhibits the tyrosine kinase activity of the receptor (Davis & Czech, 1985b). Amiloride has also been shown to inhibit cyclic AMP-dependent protein kinase, phosphorylase kinase, calmodulin-dependent glycogen synthase kinase, glycogen synthase kinase 3, glycogen synthase kinase 4, glycogen synthase kinase 5 and casein kinase 1 (Holland *et al.*, 1983). The effect of amiloride to act as a protein kinase inhibitor is consistent with its ability to decrease the phosphorylation state of the EGF receptor in intact A431 cells. The relative insensitivity of the phosphorylation state of threonine-654 to amiloride suggests that the kinase that phosphorylates this site, C-kinase (Cochet *et al.*, 1984; Hunter *et al.*, 1984; Davis & Czech, 1985a), may not be inhibited by amiloride. However, we have found that a partially purified preparation of C-kinase isolated from rat brain is inhibited by amiloride (R. J. Davis & M. P. Czech, unpublished work). Alternative hypotheses to account for the selective effect of amiloride include: (a) interaction of amiloride with phosphatases, (b) the activity of C-kinase in A431 cells in the presence of PMA may be sufficiently high that amiloride does not cause a significant inhibition, or (c) amiloride interacts directly with the EGF receptor and alters its ability to serve as a substrate for other protein kinases and phosphatases.

The results reported in the present paper indicate that most of the effect of PMA to increase the phosphorylation state of the EGF receptor can be dissociated from the effect of PMA to inhibit the high-affinity binding of ^{125}I -EGF. We conclude that the phosphorylation state of these sites on the EGF receptor does not correlate with the ability of PMA to decrease the apparent affinity of the EGF receptor. The mechanism of receptor modulation by phosphorylation at threonine-654 is an important issue for further experimentation.

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