

# Epidermal Growth Factor (EGF) Promotes Phosphorylation at Threonine-654 of the EGF Receptor: Possible Role of Protein Kinase C in Homologous Regulation of the EGF Receptor

Brian Whiteley and Luis Glaser

Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

**Abstract.** Treatment of cells with tumor-promoting phorbol diesters, which causes activation of protein kinase C, leads to phosphorylation of the epidermal growth factor (EGF) receptor at threonine-654. Addition of phorbol diesters to intact cells causes inhibition of the EGF-induced tyrosine-protein kinase activity of the EGF receptor and it has been suggested that this effect of phorbol diesters is mediated by the phosphorylation of the receptor by protein kinase C. We measured the activity of protein kinase C in A431 cells by determining the incorporation of [<sup>32</sup>P]phosphate into peptides containing threonine-654 obtained by trypsin digestion of EGF receptors. After 3 h of exposure to serum-free medium, A431 cells had no detectable protein kinase C activity. Addition of EGF to these cells resulted in [<sup>32</sup>P] incorporation into threonine-654 as well as into tyrosine residues. This indicates that EGF promotes the activation of protein kinase C in A431 cells. The phosphorylation of threonine-654 induced by EGF was

maximal after only 5 min of EGF addition and the [<sup>32</sup>P] incorporation into threonine-654 reached 50% of the [<sup>32</sup>P] in a tyrosine-containing peptide. This indicates that a significant percentage of the total EGF receptors are phosphorylated by protein kinase C. A variety of external stimuli activate Na<sup>+</sup>/H<sup>+</sup> exchange, including EGF, phorbol diesters, and hypertonicity. To ascertain whether activation of protein kinase C is an intracellular common effector of all of these systems, we measured the activity of protein kinase C after exposure of A431 cells to hyperosmotic conditions and observed no effect on phosphorylation of threonine-654, therefore, activation of Na<sup>+</sup>/H<sup>+</sup> exchange by hypertonic medium is independent of protein kinase C activity. Since stimulation of protein kinase C by phorbol diesters results in a decrease in EGF receptor activity, the stimulation of protein kinase C activity by addition of EGF to A431 cells contributes to a feedback mechanism which results in the attenuation of EGF receptor function.

**T**UMOR-PROMOTING phorbol diesters cause rapid and significant alterations of epidermal growth factor (EGF)<sup>1</sup> receptor biochemistry in intact cells. Within minutes of phorbol 12-myristate 13-acetate (PMA) addition to human epidermoid carcinoma A431 cells (39) or mouse fibroblast Swiss 3T3 cells (4), the apparent affinity of the EGF receptor decreases and the EGF-induced tyrosine-specific protein kinase activity of the cytoplasmic domain of the receptor is attenuated (9, 12). This has been associated with rapid phosphorylation of EGF receptors at serine and threonine residues (8, 9, 24).

Biochemical investigations suggest that the Ca<sup>2+</sup>- and phospholipid-dependent protein kinase (protein kinase C) is the major target for PMA action (for review see reference 33)

1. *Abbreviations used in this paper:* DAG, diacylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PMA, phorbol 12-myristate 13-acetate.

and constitutes the cellular phorbol diester "receptor" (1, 30, 32). Since purified protein kinase C phosphorylates the same residues of the EGF receptor as are found in PMA-treated cells (9), it is likely that protein kinase C catalyzes the phosphorylation in both instances. Analysis of tryptic phosphopeptides from PMA-treated EGF receptors has revealed that a major phosphorylation site is threonine-654 (12, 23), which is situated only 9 amino acids from the predicted cytoplasmic face of the plasma membrane. The phosphorylation of threonine-654 may mediate the effects of protein kinase C on binding affinity and tyrosine kinase activity because of its location between the extracellular EGF binding domain and the cytoplasmic tyrosine kinase domain. It is not known how the binding domain communicates to the cytoplasmic domain, but since there is a single membrane-spanning sequence in the EGF receptor, a site near the inner face of the plasma membrane could be a potential point of

regulation. We have demonstrated previously that addition of PMA to cells blocks the activation of  $\text{Na}^+/\text{H}^+$  exchange by EGF, platelet-derived growth factor (PDGF), and serum, but not by hypertonicity (44, 45). We postulated that a major function of protein kinase C was to modulate (inhibit) the function of the cell surface receptors for EGF and PDGF. If protein kinase C is activated in vivo as a consequence of mitogen addition to cells, a feedback loop would be established to control the intracellular events associated with mitogen addition to cells.

Protein kinase C is activated by diacylglycerols (DAGs) in vitro (27) which appear to be endogenous analogs of phorbol diesters (18). Stimulation of phosphoinositide hydrolysis and thus liberation of DAG is believed to be the physiological mechanism whereby protein kinase C is activated by mitogens (33). Phosphoinositide turnover has been demonstrated to occur in quiescent Swiss 3T3 cells stimulated with PDGF (20) and in A431 cells upon EGF addition (38, 41). The possibility exists that protein kinase C activation in mitogen-stimulated cells could be exerting the same influence on mitogen receptors as has been observed in PMA-treated cells. Evidence for physiological activation of protein kinase C should include not only data on DAG levels but also some direct measurement of phosphate incorporation resulting from protein kinase C action. Because phorbol diesters are very poorly metabolized by cells and cause intense, sustained stimulation, some estimation of the relative degree of phosphorylation which results from a physiological stimulus should aid in the ability to judge whether protein kinase C can cause negative modulation of mitogen receptors in the absence of phorbol diesters.

We have investigated the consequences of EGF addition to human epidermoid carcinoma A431 cells. We have quantitated the amounts of cellular DAG in the presence or absence of EGF and have analyzed the pattern of phosphorylation of the EGF receptor caused by PMA or EGF. We present evidence that EGF receptors are phosphorylated at threonine-654 as a result of EGF stimulation presumably as a consequence of activation of protein kinase C. Hypertonic solutions, which potently activate  $\text{Na}^+/\text{H}^+$  exchange in A431 cells, do not stimulate phosphorylation of EGF receptors at threonine-654. We conclude that this mode of activation of  $\text{Na}^+/\text{H}^+$  exchange is independent of protein kinase C. The stimulation of events which are known to attenuate receptor activity by the binding of EGF to the EGF receptor implies that protein kinase C may be contributing to a feedback mechanism in A431 cells.

## Materials and Methods

Phorbol derivatives were purchased from LC Services Corp. (Woburn, MA) and stored as stock solutions in DMSO at  $-20^\circ\text{C}$ . Mouse monoclonal antibody to the EGF receptor was purchased from Oncor (Gaithersburg, MD).  $[2\text{-}^3\text{H}]\text{Glycerol}$  and  $[^{32}\text{P}]\text{orthophosphate}$  were from New England Nuclear (Boston, MA);  $[4,5\text{-}^3\text{H}]\text{leucine}$  and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  were from Amersham Corp. (Arlington Heights, IL). 1,2-Dioleoylglycerol and 1,2-dioleoylphosphatidic acid were purchased from Sigma Chemical Co. (St. Louis, MO); formalin-fixed *Staphylococcus aureus* cells were from Bethesda Research Laboratories (Gaithersburg, MD); goat antibodies (IgG fraction) directed against mouse IgG (heavy and light chain specific) were purchased from CooperBiomedical Inc. (Malvern, PA), and Macherey-Nagel plastic-backed silica gel G sheets were from Brinkmann Instruments Co. (Westbury, NY). L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold,

NJ). Sources of other reagents and all tissue culture methods have been described (34, 35). EGF was prepared by the method of Savage et al. (37).

## Assay of Diacylglycerol

The measurement of DAG in lipid extracts from stimulated or unstimulated cells was performed by incubating lipid and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  with bacterial DAG kinase using a modification of the method of Kennerly et al. (26). A431 cells were grown on  $2 \times 1\text{-cm}$  glass slides, labeled for 24 h in medium containing  $1 \mu\text{Ci/ml}$   $[^3\text{H}]\text{glycerol}$  and switched for the final 4 h to serum-free Dulbecco's modified Eagle's medium (DME) containing  $1 \mu\text{Ci/ml}$   $[^3\text{H}]\text{glycerol}$ . Lipid extracts of the cells were obtained using a modification of the method of Bligh and Dyer (2). Each glass slide was exposed to EGF or control and placed in 5% TCA at  $4^\circ\text{C}$  to stop cellular metabolism. This step was found to be essential for rapid inactivation of the cellular enzymes that generate DAG. Direct solvent extraction of the cells gave higher basal levels of cellular DAG, which presumably resulted from inefficient enzyme inactivation. Each TCA-treated glass slide was placed in a  $13 \times 100$  test tube containing 3 ml  $\text{CHCl}_3/\text{MeOH}/0.1 \text{ N HCl}$  (1:2:0.75). After 30 min, the slide was removed and 1 ml  $\text{CHCl}_3$  plus 1 ml 0.1 N HCl was added and thoroughly mixed. The two phases were separated by centrifugation, the upper, aqueous phase was removed by aspiration, and the lower phase was taken to dryness under  $\text{N}_2$ . The lipid extracts were resuspended in 200  $\mu\text{l}$   $\text{CHCl}_3$ ; duplicate 10- $\mu\text{l}$  aliquots were placed in 12-ml conical centrifuge tubes for the assay with DAG kinase and duplicate 50- $\mu\text{l}$  aliquots were analyzed for  $[^3\text{H}]$ . Phosphorylation of DAG was performed in 25  $\mu\text{l}$  of an aqueous solution containing 0.1% Cutscum detergent (vol/vol), 30 mM  $\text{MgCl}_2$ , 105 mM NaCl, and 30 mM morpholinopropane sulfonic acid (MOPS)/NaOH, pH 7.2, with 1 mM  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (800 Ci/mol). The reaction was started by the addition of a membrane preparation from *Escherichia coli* B containing DAG kinase (26). After 15 min at  $37^\circ\text{C}$ , the reaction was terminated by the addition of 1 ml  $\text{CHCl}_3/\text{MeOH}/0.1 \text{ N HCl}$ . After 10 min at room temperature, two phases were obtained by the addition of 0.5 ml 0.1 N HCl and 0.5 ml  $\text{CHCl}_3$  containing 5  $\mu\text{g}$  phosphatidic acid as a carrier. The upper phase was removed by aspiration and the lower phase was washed by the addition of 2 ml 0.1 N HCl; the phases were separated by centrifugation and the upper phase was removed by aspiration. The lower, organic phase was taken to dryness under  $\text{N}_2$ , dissolved in  $\text{CHCl}_3$  and spotted on silica gel G thin layer sheets. The samples were resolved by a double one-dimensional thin layer chromatography system because of the presence of detergent which caused tailing (26). Spots corresponding to  $[^{32}\text{P}]\text{phosphatidic acid}$  were revealed by autoradiography and counted by liquid scintillation spectroscopy. The principal modifications of the method of Kennerly et al. (26) were (a) the use of TCA to rapidly stop metabolic activity; (b) the use of  $[^3\text{H}]\text{glycerol}$  incorporation into lipid to normalize for varying cell numbers growing on the glass chips and for recovery of cellular lipid by solvent extraction; (c) the omission of silicic acid column chromatography of lipid extracts which resulted in high variability; (d) the decrease in total lipid being added to the DAG kinase assay which prevents nonspecific inhibition of the kinase and is compensated by a fourfold increase in specific activity of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ; and (e) the substitution of plastic-backed sheets for glass plates to simplify the quantification of  $[^{32}\text{P}]$  in spots corresponding to phosphatidic acid on thin layer sheets.

A separate measurement of  $[^3\text{H}]\text{lipid}/10^6$  cells was made from cells labeled as above but trypsinized and counted with a Coulter counter. Lipid was extracted from duplicate 0.6-ml aliquots of the trypsinized cell solution by the addition of 2.4 ml  $\text{CHCl}_3/\text{MeOH}/12 \text{ N HCl}$  (2:1:0.005); separation of the phases was achieved by the addition of 1 ml  $\text{CHCl}_3$  plus 1 ml 0.1 N HCl and centrifugation. The upper phase was removed by suction aspiration and the lower phase was evaporated under  $\text{N}_2$ .  $[^3\text{H}]$  in lipid was measured by liquid scintillation spectroscopy.

## EGF Receptor Phosphorylation

EGF receptors were isolated by immunoprecipitation from detergent-solubilized A431 cells.  $[^{32}\text{P}]$ -labeled tryptic peptides were separated by a modification of the method of Davis and Czech (12). A431 cells were plated in 60-mm dishes and grown until just confluent. 24 h before use, the cells were labeled in 2 ml with  $100 \mu\text{Ci/ml}$   $[^3\text{H}]\text{leucine}$  (to normalize the results to protein in the EGF receptor) in DME containing 10% serum; 3 h before use, the cells were washed twice and placed in 2 ml serum-free DME containing  $10 \mu\text{M}$   $^{32}\text{P}$ , (1 mCi/ml) and  $100 \mu\text{Ci/ml}$   $[^3\text{H}]\text{leucine}$ . After exposure to EGF, PMA, or other treatments, cells were washed rapidly with PBS at  $37^\circ\text{C}$  and then solubilized with 1 ml of 1.5% Triton X-100/1% sodium deoxycholate/0.1%  $\text{NaDodSO}_4/0.5 \text{ M NaCl}/5 \text{ mM ethylene-diamine tetraacetic}$

acid/50 mM NaF/100  $\mu$ M sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride /10  $\mu$ g leupeptin per ml/25 mM Hepes, pH 7.8 at 4°C (12). Cell lysates were centrifuged for 1 h at 100,000 g and immunoprecipitation was achieved by the addition of 20  $\mu$ l anti-EGF receptor per ml of supernatant with end-over-end mixing at 4°C for 2 h, followed by the addition of 200  $\mu$ l formalin-fixed *S. aureus* (coated with goat anti-mouse IgG) per ml cell lysate, which was mixed for an additional 2 h at 4°C. The immunoprecipitate was washed twice by centrifugation through PBS/0.5% Triton X-100/1 M NaCl/1 M sucrose, and receptors were dissociated by boiling 15 min in 60  $\mu$ l 5% NaDodSO<sub>4</sub>/20 mM dithiothreitol followed by centrifugation. The receptors were reduced and alkylated by the addition of 30  $\mu$ l 0.4 M iodoacetamide/50 mM Tris, pH 8.6, for 15 min at room temperature followed by the addition of 25  $\mu$ l 25% 2-mercaptoethanol/25% glycerol/50% 50 mM Tris, pH 8.6, and incubation for 15 min at 60°C. The proteins were then separated by electrophoresis on a 7% NaDodSO<sub>4</sub>/polyacrylamide gel by the method of Laemmli (29).

After electrophoresis, the [<sup>32</sup>P]-labeled EGF receptors were located by autoradiography and the appropriate regions of the gel were washed with three changes of 85% acetone/5% acetic acid/5% triethylamine/5% H<sub>2</sub>O to remove the NaDodSO<sub>4</sub>. The gel slices were digested at 37°C with 50  $\mu$ g/ml TPCK-treated trypsin in 3 ml of 50 mM NH<sub>4</sub>HCO<sub>3</sub>. After 14 h, a second addition of trypsin was made and the digestion was allowed to proceed for a total of 24 h. The [<sup>32</sup>P]-labeled phosphopeptides were lyophilized, dissolved in 300  $\mu$ l of 1% trifluoroacetic acid, and 50  $\mu$ l was used to determine [<sup>3</sup>H] content as a measure of EGF receptor protein. The peptides in 250  $\mu$ l were resolved by reverse-phase HPLC using a Waters  $\mu$ Bondapak C<sub>18</sub> column equilibrated with 0.1% trifluoroacetic acid. The column was washed for 5 min and then the phosphopeptides were eluted by a linear gradient of acetonitrile (0–55%) over 55 min. The flow rate was 1 ml/min and fractions were collected every 1 min. The [<sup>32</sup>P]phosphopeptides were detected by measuring Cerenkov radiation. Phosphoamino acid analysis was performed by the method of Hunter and Sefton (21).

### Preparation of Synthetic Phosphopeptides

The synthetic pentapeptide, Lys-Arg-Thr-Leu-Arg, used as a reference, was kindly prepared by Mr. Joseph Leykam (Washington University, St. Louis, MO) and the sequence was confirmed by automated Edman degradation. The peptide was phosphorylated using the catalytic subunit of the cAMP-dependent protein kinase (a gift from Dr. Linda Pike, Washington University, St. Louis, MO) in a mixture containing 200  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP, 10 mM MgCl<sub>2</sub>, 20 mM 2-mercaptoethanol, and 40 mM Tris, pH 6.8. After incubation for 2 h at 37°C, bovine serum albumin was added as a protein carrier and protein was precipitated by the addition of concentrated TCA to a final concentration of 5% (wt/vol). After centrifugation, the supernatant was spotted on 2- × 2-cm squares of phosphocellulose paper and after drying, was washed with four changes of 75 mM phosphoric acid. The phosphopeptide was finally eluted with 0.5 M ammonium formate, pH 10, and lyophilized before purification by reverse-phase HPLC.

## Results

### EGF-stimulated DAG Generation

Sawyer and Cohen (38) reported that EGF-stimulated phosphatidylinositol (PI) turnover in A431 cells, which they measured as [<sup>32</sup>P]phosphate or [<sup>3</sup>H]inositol incorporation into PI. They suggested that protein kinase C could be activated in EGF-stimulated cells by inferring that increased DAG levels are a consequence of the observed increase in PI turnover. DAG is very rapidly metabolized to phosphatidic acid in most cells, therefore only direct measurements of the concentrations of DAG are likely to be relevant to the activity of protein kinase C. We have modified the method of Kennerly et al. (26) to be able to rapidly measure the levels of DAG in cells. One of the most important modifications is rapid inactivation of cellular metabolism before lipid extraction. For example, direct extraction of cells without prior treatment with TCA resulted in a 40% increase in DAG and solubilization of cells in NaDodSO<sub>4</sub> at 4°C before extrac-

tion resulted in DAG levels which were 2.7-fold greater than DAG levels from acid-treated cells.

In Table I we show the amounts of DAG in cells treated with EGF. A modest increase is observed 2 min after addition of EGF and this increase remained elevated after 10 min. The absolute values of DAG are, however, dependent on the prior treatment of cells; for example, length of exposure of cells to 1% serum. In no case did we observe EGF-induced increases in DAG levels greater than 45%. The modest increases in DAG levels makes it extremely difficult to interpret these observations in terms of protein kinase C activity, since the cellular location of the DAG is not known. Note that the increase in DAG concentration is much less than what would be inferred from a sixfold increase in the rate of PI turnover (38).

### Identification of the Phosphopeptides Containing Threonine-654

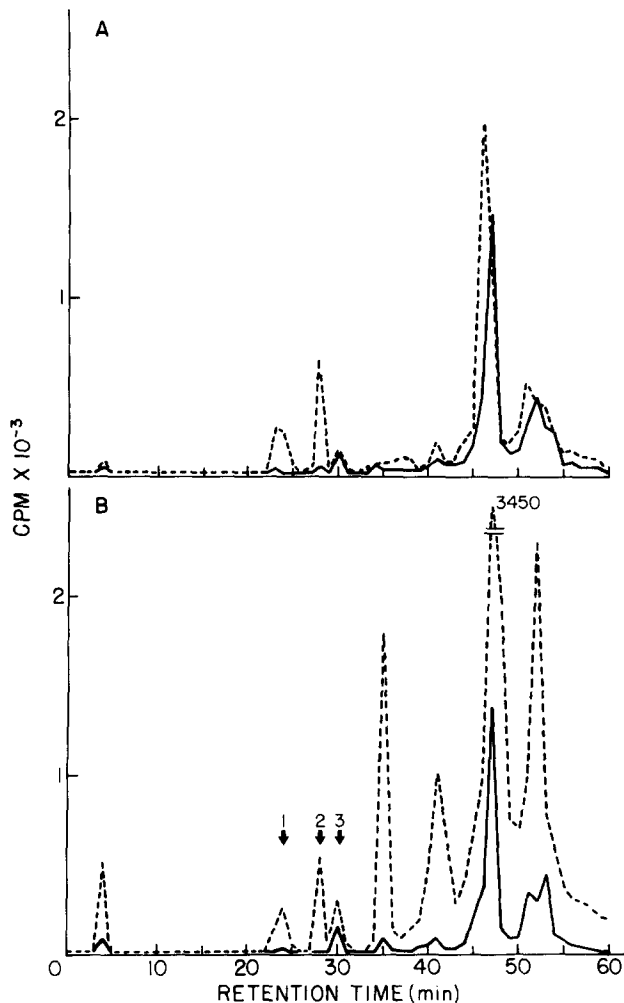
Davis and Czech (12) reported that tryptic phosphopeptides derived from the EGF receptor could be resolved by reverse-phase HPLC analysis and that two [<sup>32</sup>P]-labeled phosphopeptides containing threonine-654 and eluting at low acetonitrile concentrations could be identified in tryptic digests of the EGF receptor derived from PMA-treated cells. They designated the two phosphopeptides, "1" and "2", and identified "2" as the pentapeptide, Lys-Arg-Thr(P)-Leu-Arg. They reported that trypsin digestion slowly converted "2" to the tetrapeptide Arg-Thr(P)-Leu-Arg, which was designated as peptide "1"; the numbers represent the order of their elution by reverse-phase HPLC. The tryptic maps of EGF receptors derived from PMA-treated and control cells is shown in Fig. 1 A. In every chromatogram, a peak of varying size is observed at 4 min, which represents free [<sup>32</sup>P]P<sub>i</sub>. The next two peaks, which elute at 24 and 28 min, correspond to the phosphopeptides "1" and "2" of Davis and Czech (12). A third peak elutes at 30 min and is seen in control cells as well as PMA-treated cells. The only other characteristic feature of Fig. 1 A is a large peak eluting at ~46 min which appears to represent stable site(s) of phosphorylation because [<sup>32</sup>P] in control cells is predominantly found in this phosphopeptide. Fig. 1 B demonstrates that the two peaks, "1" and "2", of Davis and Czech (12) are present in tryptic maps of the EGF receptor obtained from EGF-stimulated cells as well as PMA-treated cells. Phosphoamino acid analysis of peaks 1, 2, and 3 from EGF-stimulated cells (Fig. 1 B) demonstrates that peaks 1 and 2 contain phosphothreonine while peak 3 contains phosphoserine (see Fig. 3). Phosphoamino acid analysis of the phosphopeptides 1, 2,

Table I. Effect of EGF Addition on the Concentration of DAG in A431 Cells

Time after EGF addition	DAG	Normalized value
min	pmol/10 <sup>6</sup> cells	
0	1,982 (± 149)*	1.00
2	2,523 (± 173)	1.27
10	2,584 (± 187)	1.30

A431 cells were grown as described in Materials and Methods; after exposure of cells to EGF (100 ng/ml), lipids were extracted from cells and DAG concentration was determined.

\*SEM, n = 3.



**Figure 1.** Phosphopeptide maps of the EGF receptor from A431 cells. (A) EGF receptors derived from control cells (solid line) and cells treated with 10 nM PMA for 15 min (dashed line). (B) EGF receptors derived from control cells (solid line) and cells exposed to 200 ng/ml EGF for 15 min (dashed line). Trypsin digestion and HPLC were as described in Materials and Methods. The peaks labeled 1, 2, and 3 in B correspond to samples in Fig. 3.

and 3 from PMA-treated cells was identical to the results found in Fig. 2 (data not shown). To confirm the assignment of Lys-Arg-Thr(P)-Leu-Arg as peptide 2, we synthesized this pentapeptide and phosphorylated it using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as described. After trypsinization of the synthetic phosphopeptide for 24 h with 50  $\mu\text{g}/\text{ml}$  TPCK-treated trypsin, the two phosphopeptide products were chromatographed by reverse-phase HPLC and found to co-elute with peaks 1 and 2 (Fig. 1 B) (data not shown). In all calculations, the sum of  $^{32}\text{P}$  in peaks 1 and 2 is used to measure the total phosphate incorporation into threonine-654 of the EGF receptor.

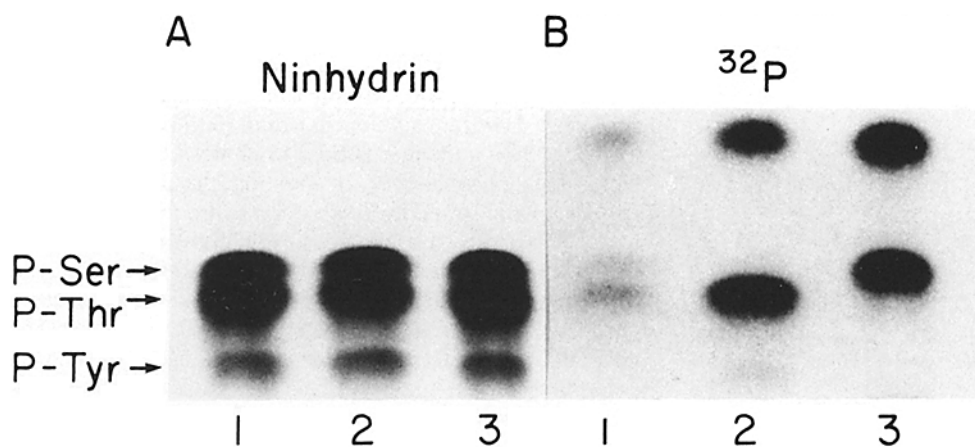
In Fig. 1 B, a sharp peak eluted at 35 min. This phosphopeptide contains phosphotyrosine (data not shown) and presumably represents a single site of autophosphorylation of the receptor after treatment with EGF. The  $^{32}\text{P}$  incorporated into this phosphopeptide is used as a reference in calculations designed to estimate the relative amount of threonine-654 phosphorylation.

### **Effect of EGF on the Phosphorylation of Threonine-654**

Fig. 1 b is a peptide map of the EGF receptor from  $^{32}\text{P}_i$ -labeled cells showing peaks 1 and 2 which represent Arg-Thr(P)-Leu-Arg and Lys-Arg-Thr(P)-Leu-Arg, respectively. The two phosphopeptides, known to be produced by PMA treatment (12) are also generated by EGF addition to A431 cells (Fig. 1 b). The time course of phosphopeptide formation after the addition of EGF is shown in Fig. 3. The phosphorylation of threonine-654 was detected as early as 1 min after EGF addition and maximal phosphate incorporation seemed to occur 5 min after EGF addition. EGF stimulates substantial phosphorylation of tyrosine in EGF receptors and one phosphotyrosine-containing tryptic peptide is found to elute at 30% acetonitrile (fraction 35) during reverse-phase HPLC. The identity of phosphotyrosine in this peptide was confirmed by phosphoamino acid analysis (data not shown). To estimate the extent of threonine-654 phosphorylation in EGF-stimulated cells, the fraction of  $^{32}\text{P}$  incorporated into peaks 1 and 2 relative to  $^{32}\text{P}$  incorporated into the phosphotyrosine-containing peptide at fraction 35 was calculated. Using this method, in two experiments  $^{32}\text{P}$  incorporation into threonine-654 was 43 and 53% of  $^{32}\text{P}$  incorporation due to autophosphorylation (Table II). In these experiments we used 200 ng/ml of EGF which gave a maximal biological response in these cells (22, 35). The phosphotyrosine peptide eluted at fraction 35 appears to represent the major phosphotyrosine autophosphorylation site in the EGF receptor, at tyrosine 1173 (14). If we assume that 200 ng/ml EGF-stimulated autophosphorylation in essentially 100% of the EGF receptors then the values in Table II would allow us to estimate the percentage of receptors which have been phosphorylated at threonine-654. Our conclusion is that 40–50% of the EGF receptors may be modified by the action of EGF-stimulated protein kinase C.

### **Phosphorylation of EGF Receptors by Exposure to Hypertonic Medium**

Exposure of A431 cells to medium of increased osmolarity leads to activation of  $\text{Na}^+/\text{H}^+$  exchange and to an increase in intracellular pH which displays similar kinetics to that observed upon addition of mitogens to the cells (6).  $\text{Na}^+/\text{H}^+$  exchange also appears to be activated during the regulatory volume increase response in certain epithelial cells (16) and nucleated erythrocytes (5, 28). Whereas  $\text{Na}^+/\text{H}^+$  exchange acts to restore epithelial cell and erythrocyte volume after exposure to hypertonic solutions, A431 cell volume is not significantly affected by  $\text{Na}^+/\text{H}^+$  exchange activity (6). The role of protein kinase C is poorly understood during volume regulation in various cells or osmotic perturbation in A431 cells, but PMA has been observed to exert effects similar to osmotic perturbation (19) which has led to speculation that the activity of protein kinase C mediates volume regulation in certain cells. Fig. 4 shows the HPLC profile of EGF receptor tryptic phosphopeptides from A431 cells exposed for 30 min to medium made 200 mM hypertonic by the addition of 2 M sucrose (200  $\mu\text{l}$  into 2 ml isotonic medium). There was no difference from control at the positions of the phosphopeptides 1 and 2, which correspond to  $^{32}\text{P}$ -labeled threonine-654. This implies that protein kinase C is not activated by exposure to hypertonic medium, a treatment which results in potent stimulation of  $\text{Na}^+/\text{H}^+$  exchange. An alter-



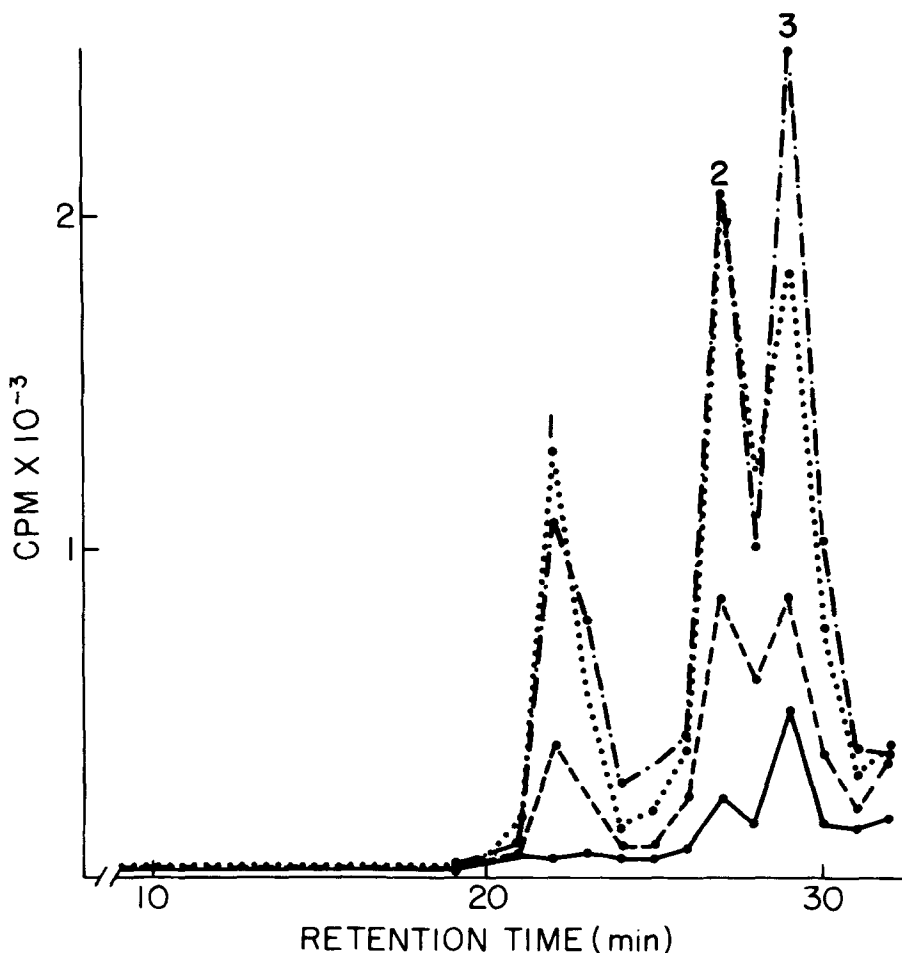
**Figure 2.** Phosphoamino acid analysis of peptides from EGF receptor. The peptides were obtained from EGF receptors, derived from cells to which EGF was added for 15 min and are identified by numbers as in Fig. 1 B. A shows ninhydrin staining of samples and indicates the positions of phosphoamino acid standards after electrophoresis on thin-layer cellulose sheets by the method of Hunter and Sefton (21). B shows the autoradiogram of the chromatograph in A (for details see Materials and Methods).

native, albeit unlikely, explanation could be that protein kinase C is activated, but that the EGF receptor is not a substrate after osmotic perturbation. Fig. 4 also shows that 100 nM 4 $\alpha$ -PMA has no effect on [ $^{32}$ P]P $_i$  incorporation into the EGF receptor, therefore, the PMA effects are stereospecific and are consistent with the known specificity of protein kinase C activation by phorbol diesters (7).

### Discussion

The role of protein kinase C as a mediator of the response

of cells to mitogens has been the subject of considerable debate. Since phorbol diesters are mitogenic for some cells and a number of mitogens activate PI turnover it seemed reasonable to assume that protein kinase C might be responsible for the mitogenic response. As described in the introduction, detailed studies of the EGF receptor suggested that it is inactivated by phosphorylation at threonine-654, which is catalyzed by protein kinase C. Previously, we reported that exposure of cells to low concentrations of PMA causes attenuation of Na $^+$ /H $^+$  exchange activation by EGF or serum in A431 cells (44) and PDGF or serum in NR6 cells



**Figure 3.** Time course of phosphorylation of the EGF receptor at threonine-654. EGF receptors derived from control cells (solid line) and cells treated with 200 ng/ml EGF for 1 min (dashed line), 5 min (dotted line), or 15 min (dashed and dotted line). Trypsin digestion and HPLC analysis is described in Materials and Methods. The peaks labeled 1, 2, and 3 correspond to the samples in Fig. 3.

**Table II. Extent of Phosphorylation of EGF Receptors at Threonine-654**

	Time	Ratio
	<i>min</i>	
Exp. 1	1.0	0.38
	5.0	0.50
	15.0	0.53
Exp. 2	1.0	0.23
	5.0	0.42
	15.0	0.43

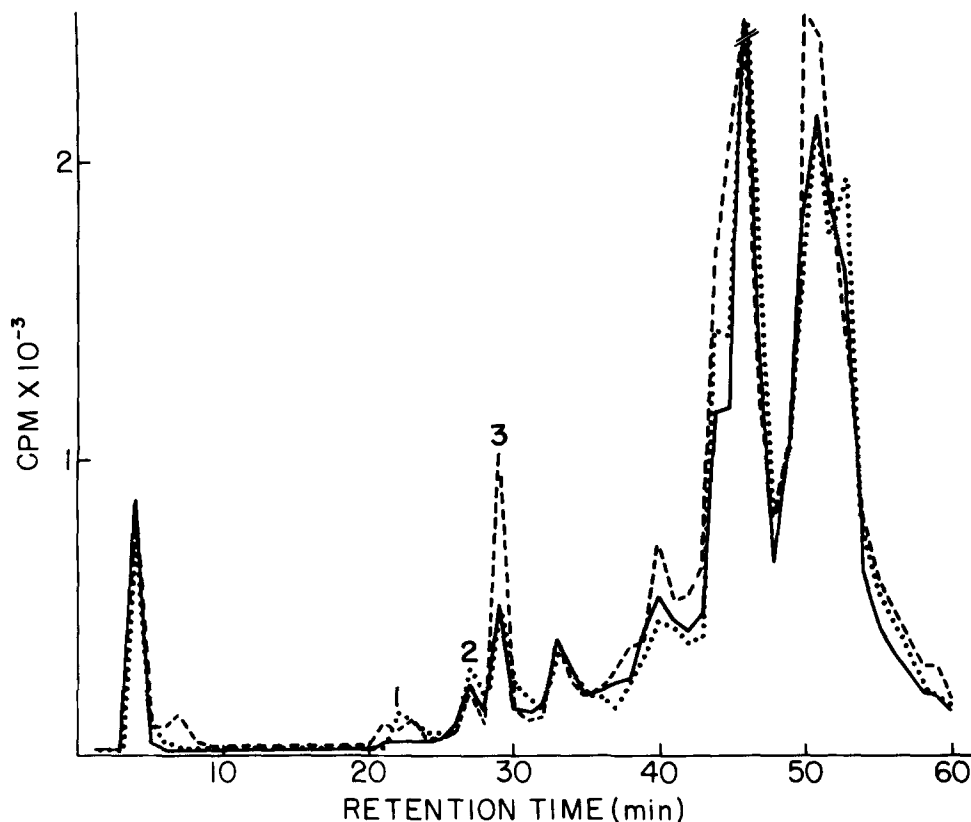
Relative phosphorylation of threonine-654 as a result of exposure of A431 cells to EGF (200 ng/ml) for varying lengths of time was determined by measuring the ratio of [<sup>32</sup>P] in threonine-654-containing peptides to the [<sup>32</sup>P] content of a tyrosine-containing peptide which eluted at 35 min (see Materials and Methods). Note that the ratio of phosphorylation in these two peptides increases between 1 and 5 min of exposure to EGF, and represents increased phosphorylation of threonine-654.

(45). The mechanism of this inhibition is not known with certainty, but could be fully explained by the inhibition of mitogen receptor tyrosine kinase activity. Phorbol diesters have also been shown to decrease phosphoinositide hydrolysis and Ca<sup>2+</sup> mobilization in thrombin-stimulated platelets (43). In Swiss 3T3 cells, the mitogens serum, PDGF, and fibroblast growth factor have been shown to stimulate a rapid, transient rise in [Ca<sup>2+</sup>] which is inhibitable by a 30-min pretreatment with PMA (31). Therefore, the ability of PMA to block mitogenic stimulation of Na<sup>+</sup>/H<sup>+</sup> exchange might also be attributable to inhibition of Ca<sup>2+</sup> mobilization or PI turnover.

Other investigators have also shown that the function of a variety of cell surface receptors for hormones or mitogens

can be "inactivated" by phorbol diesters which activate protein kinase C (25, 40, 42), suggesting that in all of these systems the PI cycle serves as part of a feedback loop to modulate the activity of these receptors. Heterologous ligands such as PDGF (3, 46), vasopressin (36), and bombesin (4) have been shown to cause decreases in EGF receptor affinity in 3T3 cells in a manner similar to phorbol diesters. All of these peptide ligands have, in common, the ability to stimulate phosphoinositide hydrolysis and cause the liberation of DAG. Therefore, the effects of PDGF, vasopressin, and bombesin on EGF receptor affinity in 3T3 cells are likely to be mediated by protein kinase C activity and the subsequent phosphorylation of threonine-654 of the EGF receptor. The modulation of the EGF receptor by heterologous ligands, an example of what might be termed "cross talk" between mitogen receptors, is an important example of the way in which protein kinase C may be functioning during mitogenic stimulation of quiescent cells. Since EGF has also been reported to cause stimulation of phosphoinositide turnover, the potential for homologous regulation of the EGF receptor is apparent. Evidence for this type of regulation must begin with measurements designed to determine whether protein kinase C activity increases after addition of EGF to intact cells.

Measurements of intracellular Ca<sup>2+</sup> or DAG levels as a way of assessing changes in protein kinase C levels are usually ambiguous since the effective concentrations of these ligands available to C-kinase are unknown. The measurement of cytoplasmic Ca<sup>2+</sup> after addition of mitogens by the use of aequorin, which may be the most reliable method currently available, suggests that increases in Ca<sup>2+</sup> may be extremely transient (31). As documented here, the determination of DAG levels (Table I) when carried out carefully only



**Figure 4.** Effect of hypertonicity on phosphorylation of EGF receptors in A431 cells. EGF receptors derived from control cells (solid line) and cells treated with 100 nM 4 $\alpha$ -PMA (dotted line) or cells exposed to hypertonic medium (dashed line). Trypsinization and HPLC analysis is described in Materials and Methods. The elution positions of phosphopeptides containing threonine-654 are indicated by the numbers 1 and 2. The data indicate that this residue is not phosphorylated under hypertonic conditions or after addition of a phorbol diester which does not activate protein kinase C.

indicated a small but possibly significant change in DAG concentration after addition of EGF to A431 cells. However, by themselves, these data cannot be used to indicate that protein kinase C is activated after addition of EGF to A431 cells.

Davis and Czech (12) have provided a direct method for assaying the activity of protein kinase C in cells with high levels of EGF receptor by measuring phosphorylation of the EGF receptor at threonine-654. We have introduced some minor modifications of this method and have controlled for receptor recovery by the use of [<sup>3</sup>H]leucine to metabolically label the EGF receptor.

Our observations suggest that protein kinase C is rapidly activated after addition of EGF to cells and remains active for substantial periods of time (at least 15 min) after addition of EGF to cells. The existence of the proposed feedback loop by which activation of protein kinase C might "inactivate" the EGF receptor is thus confirmed. However, the magnitude of the effect is hard to estimate. If, as assumed above, we use as a reference point the phosphorylation of a tyrosine residue in the receptor, we can estimate that 40–50% of the EGF receptor molecules are phosphorylated at threonine-654, a rather substantial number. We also observed that the degree of phosphorylation of threonine-654 after addition of EGF to A431 cells is comparable to that observed after addition of PMA (see Fig. 1, A and B), again suggesting that the activation of protein kinase C after addition of EGF to A431 cells is biologically significant. While the data clearly support the presence of such a feedback loop in A431 cells, its presence in other cell types remains to be determined. The high level of EGF receptors in A431 cells makes the type of measurement we have described easier, but also may magnify the response.

Recent studies using synthetic peptide substrates (15) indicate that phosphorylation of the isolated EGF receptor by protein kinase C in vitro may only reduce the tyrosine-protein kinase activity of the receptor at saturating EGF concentrations by only 20%. However, we observed that a 60-min treatment with 10<sup>-8</sup> M PMA followed by addition of EGF for 5 min caused a 70% decrease in EGF-stimulated [<sup>32</sup>P] incorporation into a phosphopeptide which is believed to contain a single tyrosine autophosphorylation site. This inhibition of tyrosine kinase activity by phorbol diesters in vivo which has been reported by others previously (17) is significantly greater than that observed by Downward et al. (15) in vitro, thus the solubilized system is somehow not representative of the interactions of protein kinase C and the EGF receptor in vivo. To accurately estimate the modulation of the EGF receptor tyrosine kinase activity by EGF-induced protein kinase C activity in the absence of other metabolic events, it would be necessary to measure autophosphorylation in the presence or absence of protein kinase C in intact cells. Unfortunately, the compounds which have been shown to inhibit the activity of protein kinase C in vitro are too nonspecific or are ineffective in vivo. Furthermore, the technique of down-regulating protein kinase C by chronic exposure of cells to high concentrations of phorbol diesters (11) has recently been shown to cause a change in substrate specificity rather than a decrease in total activity of the enzyme in some cell types (10). A 24-h exposure to 400 nM PMA does not significantly decrease the activity of protein kinase C in A431 cells (Whiteley, B., unpublished observations).

Continuous recording of intracellular pH after mitogen addition to A431 cells has revealed that EGF or serum induces an amiloride-sensitive pH<sub>i</sub> increase after a lag period of ~2 min (35). Intracellular pH increases to a new steady state, but then returns to the initial prestimulatory level 30–60 min after mitogen addition (Whiteley, B., unpublished observations). This transient nature of intracellular alkalization might also be due to negative modulation of the stimulation by the EGF receptor of Na<sup>+</sup>/H<sup>+</sup> exchange, which could be mediated by protein kinase C.

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