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Phosphorylation at threonine-654 is not required for negative regulation of the epidermal growth factor receptor by non-phorbol tumor promoters

(thapsigargin/calcium ionophore/protein kinase C)

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ABSTRACT **Phosphorylation of the epidermal growth factor (EGF) receptor following activation of protein kinase C appears to negatively regulate EGF binding and the receptor-associated tyrosine kinase activity. We have identified two agents, the calcium ionophore A23187 and the non-phorbol tumor promoter thapsigargin, that similarly inhibit the EGF receptor binding and kinase activities through protein kinase C-independent pathways. Both agents activate protein kinases that phosphorylate the EGF receptor in A431 cells. To test the hypothesis that negative regulation of the EGF receptor always occurs through phosphorylation of threonine-654, a site uniquely phosphorylated by protein kinase C, we analyzed the tryptic phosphopeptides of EGF receptors isolated from cells treated with these agents. While limited phosphorylation of threonine-654 results from the A23187 treatment, no significant phosphorylation of this residue is detected after thapsigargin treatment. These results suggest that EGF receptor phosphorylation is a general mechanism for altering receptor properties and that site(s) of phosphorylation other than threonine-654 may negatively regulate the kinase activity as well as the binding of the EGF receptor.**

The epidermal growth factor (EGF) receptor is a tyrosine kinase that is utilized by a variety of cells to mediate cell proliferation. There are several lines of evidence that suggest that activation of the tyrosine kinase of the EGF receptor is essential for triggering a mitogenic response. When this tyrosine kinase activity is abolished by site-directed mutagenesis, EGF fails to stimulate cell division (1). Conversely, there is a strong correlation between constitutive activation of this tyrosine kinase activity and cell transformation. Constitutive activation can result from truncation of the EGF receptor or autocrine or paracrine stimulation of the EGF receptor by EGF or transforming growth factor type α.

In normal cells, the EGF receptor tyrosine kinase appears to be tightly controlled. Activation not only requires EGF binding but is dependent on the state of phosphorylation of the EGF receptor. Autophosphorylation of tyrosine residues appears to play a role in activation of the receptor-associated tyrosine kinase activity (2). Negative control can occur through increases in serine and/or threonine phosphorylation. For example, treatment with phorbol 12-myristate 13-acetate (PMA)-type tumor promoters causes inhibition of high-affinity EGF receptor binding and inhibition of EGF-stimulated tyrosine kinase activity *in vivo* (3–6). These effects are associated with increased phosphorylation of several serine and threonine residues of the receptor.

The actual sites of phosphorylation on the EGF receptor that are responsible for regulating the biological properties of the molecule have not been resolved. One residue, threonine-654, has received much attention because it appears to be uniquely phosphorylated by activators of protein kinase C (7–9). Although there are conflicting reports in the literature, recent site-directed mutagenesis experiments suggest that threonine-654 is required for protein kinase C-dependent inhibition of the EGF receptor kinase but not EGF binding (refs. 10–12; data not shown). Since threonine-654 is neither the only site nor the major site on the EGF receptor that is phosphorylated when cells are treated with tumor promoters or other activators of protein kinase C (7, 9, 13), it is possible that alternative sites of phosphorylation are important in regulating high-affinity EGF receptor binding and/or the ability of EGF to stimulate the EGF receptor tyrosine kinase.

One approach for investigating the role of threonine-654 in EGF receptor modulation is to identify pathways other than protein kinase C that alter EGF receptor properties. Several growth factors, such as platelet-derived growth factor (PDGF) (14), factors from v-*sis* transformed cells (15), acidic fibroblast-derived growth factor (16), and basic fibroblast-derived growth factor (unpublished data) have been shown to inhibit EGF receptor binding in fibroblast cells via protein kinase C-independent pathways. We now demonstrate that two exogenous agents, the calcium ionophore A23187 and a newly discovered non-PMA-type tumor promoter thapsigargin (17, 18), are able to inhibit EGF binding and EGF-stimulated autophosphorylation of the receptor in A431 cells through mechanisms that do not involve protein kinase C activation. Our results provide evidence that phosphorylation of threonine-654 is not required for negative regulation of the EGF receptor kinase by these factors.

MATERIALS AND METHODS

Materials and Cells. Human epithelial carcinoma A431 cells (American Cell Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (GIBCO) in a gassed (5.5% CO_2/94% air), humidified atmosphere at 37°C. Phorbol 12,13-dibutyrate (PDBu) and A23187 were purchased from Sigma. The non-PMA-type tumor promoter thapsigargin was a gift from S. B. Christensen (Royal Danish School of Pharmacy, Copenhagen). EGF was purchased from Biomedical Technologies (Stoughton, MA). Monoclonal anti-phosphotyrosine antibody was a gift from A. R. Frackelton, Jr. (Brown University and Roger Williams

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Abbreviations: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; PDBu, phorbol 12,13-dibutyrate.

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Hospital, Providence, RI). Polyclonal anti-erbB antiserum was a gift from S. Decker (Rockefeller University, New York). Mouse EGF was iodinated by the chloramine-T method to a specific activity of ≈1 mCi/μmol (1 Ci = 37 GBq) using $Na^{125}I$ (Amersham).

Binding Studies. ^{125}I-labeled EGF (0.05–1 nM) was bound to high-affinity EGF receptors in A431 cells at 4°C as described (15).

Generation of Protein Kinase C-Depleted Cells. Cells were depleted of protein kinase C as described (15). Briefly, confluent A431 cells were incubated in DMEM supplemented with insulin/transferrin/selenium (Collaborative Research) containing PDBu (1 μg/ml) for 24 hr for binding studies. For phosphorylation studies, cells were incubated in DMEM minus phosphate medium containing PDBu (1 μg/ml) for an additional 24 hr prior to addition of $^{32}P_i$.

Isolation of $^{32}PO_4$-Labeled EGF Receptors. Cells were placed in DMEM minus PO_4 with or without PDBu (1 μg/ml) overnight and then equilibrated with $^{32}PO_4$ (0.5–4 mCi/ml) for 3–4 hr at 37°C. The cells were then treated with the appropriate agents at 37°C for the amount of time indicated in the text, and the EGF receptor was isolated by immunoprecipitation with anti-phosphotyrosine antibodies or anti-erbB antiserum as described (3, 19). Under these conditions, no ^{32}P-labeling of threonine-654 after thapsigargin treatment was detected. The EGF receptor was resolved by SDS/PAGE (7.5%) and visualized by autoradiography. To quantitate EGF receptor phosphorylation, the Cerenkov radiation associated with excised EGF receptor gel bands was determined.

HPLC Analysis of EGF Receptor Phosphotryptic Peptides. Gel slices were swollen in 0.1 mg of trypsin in 1 M NH_4HCO_2 for 6 hr at 37°C and then treated with an additional 0.1 mg of trypsin at 37°C overnight. The supernatant was lyophilized and the fractions were dissolved in 0.5% acetonitrile and 0.1% trifluoroacetic acid in distilled water for HPLC analysis. Phosphotryptic peptides were separated on a Bio-Rad RP318 column and eluted by a linear acetonitrile gradient that ranged from 0.5% to 50% acetonitrile over 80 min. The flow rate was 1.1 ml/min. The Cerenkov radiation associated with the eluted fractions was determined.

RESULTS AND DISCUSSION

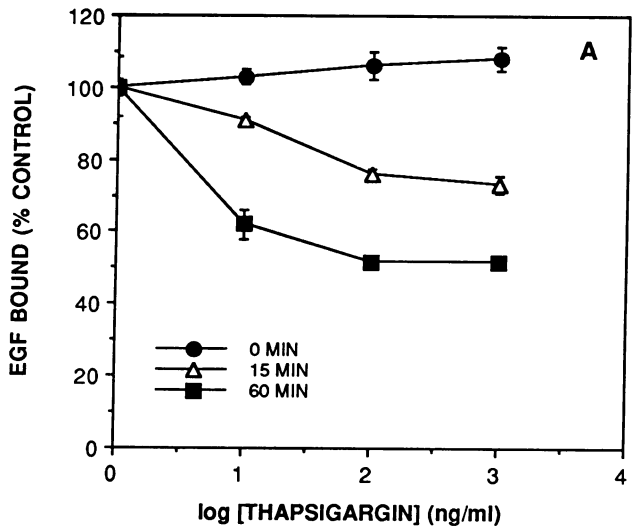
To study the mechanisms by which the EGF receptor binding and kinase activities can be regulated, we screened several growth-modulating and tumor-promoting compounds for their ability to alter EGF receptor properties independent of activation of protein kinase C. To facilitate phosphorylation studies, we used human epidermal carcinoma (A431) cells, which have high numbers of EGF receptors but lack receptors for growth factors such as PDGF and fibroblast-derived growth factor that have been shown to modulate EGF receptor properties through multiple pathways (14–16). Two agents were found to mimic the action of protein kinase C activators on the EGF receptor in A431 cells: the calcium ionophore A23187, and the non-PMA-type tumor promoter thapsigargin.

While it had been shown previously that A23187 treatment can decrease EGF binding in 3T3 cells, Rat-1 cells, and A431 cells (20), the mechanism by which increased calcium leads to decreased EGF receptor binding is not clear. To test whether this inhibition requires activation of protein kinase C, cells were treated for 48 hr with PDBu to induce loss of protein kinase C activity as described (15). Treatment with 50 μM A23187 for 5 min at 37°C inhibits high-affinity EGF binding in both control the protein kinase C-depleted A431 cells (Table 1). The dose response for inhibition is indistinguishable between control and PDBu-treated cells (data not shown).

Similar results are obtained upon treatment of A431 cells with thapsigargin, a non-PMA-type tumor promoter that shares some of the properties of the PMA-type tumor promoters. Unlike PMA, thapsigargin does not activate protein kinase C *in vitro* (unpublished data). Thapsigargin causes inhibition of high-affinity EGF binding in A431 cells, although it is less potent than PDBu (Fig. 1). Like the calcium

Table 1. High-affinity EGF receptor binding in control and protein kinase C-depleted A431 cells

Treatment	^{125}I-labeled EGF binding, cpm	
	Control A431 cells	Protein kinase C-depleted A431 cells
Control medium	7,856 ± 10	7,754 ± 592
A23187 (50 μM; 5 min; 37°C)	5,501 ± 588	5,238 ± 124
Control medium	10,788 ± 396	9,606 ± 55
Thapsigargin (100 ng/ml; 60 min; 37°C)	7,279 ± 159	6,888 ± 285
Control medium	10,843 ± 249	10,613 ± 128
PDBu (100 ng/ml; 60 min; 37°C)	7,050 ± 193	10,643 ± 425



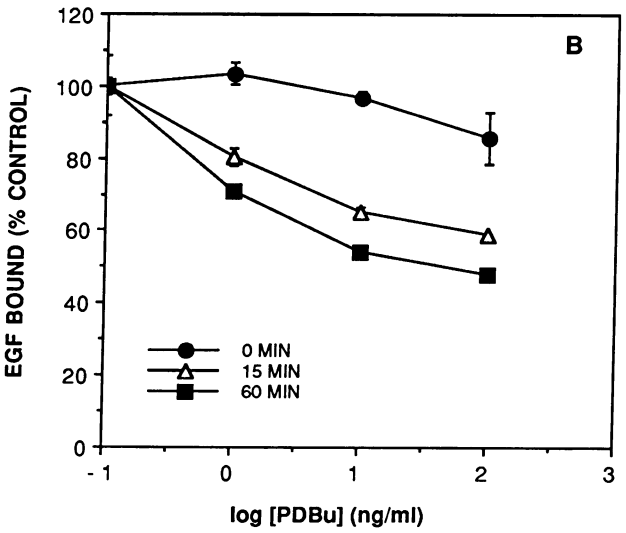


FIG. 1. Thapsigargin treatment of A431 cells causes inhibition of high-affinity EGF receptor binding. Confluent A431 cells were exposed to thapsigargin (*A*) or PDBu (*B*) at 37°C at the indicated doses for 0 min (●), 15 min (△), or 60 min (■). Cells were then assayed for ^{125}I-labeled EGF binding. Total EGF binding (100%) was 8438 ± 699 cpm. Nonspecific binding was 241 cpm.

ionophore A23187 but unlike the PMA-type tumor promoters, thapsigargin is equally effective at inhibiting EGF binding in cells depleted of protein kinase C at all doses tested (Table 1; data not shown).

Inhibition of high-affinity EGF binding by activators of protein kinase C is associated with loss of EGF-stimulated tyrosine kinase activity as measured either *in vivo* by EGF receptor autophosphorylation (3) or *in vitro* by phosphorylation of exogenous substrates (4). In A431 cells, the major *in vivo* substrate for the EGF-stimulated kinase is the EGF receptor itself (3). To test whether loss of high-affinity binding induced by protein kinase C-independent pathways also results in loss of EGF-stimulated tyrosine kinase activity, we immunoprecipitated tyrosine phosphorylated EGF receptor from cells labeled with $^{32}P_i$ by using a high-affinity anti-phosphotyrosine monoclonal antibody (21).

When cells are treated with A23187 at concentrations that inhibit high-affinity EGF binding, EGF-stimulated tyrosine phosphorylation of the EGF receptor is reduced (Fig. 2*A*). This result is very similar to the effect of PDBu on EGF-stimulated tyrosine phosphorylation (Fig. 2*A*; ref. 3). However, unlike PDBu, the calcium ionophore also inhibits EGF-stimulated receptor tyrosine phosphorylation in cells that have been depleted of protein kinase C (Fig. 2*B*). Similar results are obtained after treatment of cells with thapsigargin, which inhibits EGF-stimulated receptor autophosphorylation in both control (data not shown) and protein kinase C-depleted cells (Fig. 2*C*). Thus, like the action of the calcium ionophore, the inhibitory effects of thapsigargin are observed independent of the level of active protein kinase C. The fact that high-affinity EGF receptor binding and EGF-stimulated autophosphorylation can be inhibited by both protein kinase C-dependent and -independent pathways raises the possibility that receptor modification is occurring through a similar mechanism.

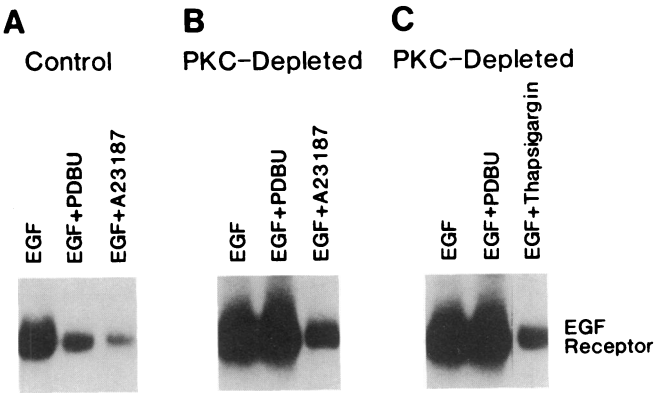


FIG. 2. The calcium ionophore A23187 and thapsigargin inhibit EGF-stimulated receptor autophosphorylation in A431 cells. (*A*) Calcium ionophore treatment of normal cells. Confluent A431 cells were labeled with $^{32}P_i$ and then incubated in the absence (EGF) or presence of PDBu (1 μg/ml) (EGF + PDBu) or 50 μM A23187 (EGF + A23187) for 5 min at 37°C. EGF (150 ng/ml) was then added for an additional 5 min, and the EGF receptors were isolated by monoclonal anti-phosphotyrosine antibody affinity chromatography and SDS/PAGE. In the absence of EGF, no significant phosphorylation of the EGF receptor is detected (3). (*B*) Calcium ionophore treatment of protein kinase C (PKC)-depleted cells. Cells were depleted of protein kinase C and then labeled and treated as in *A*. (*C*) Thapsigargin treatment of protein kinase C-depleted cells. Cells were depleted of protein kinase C and labeled as in *B*. After labeling, cells were incubated in the absence (EGF) or presence of PDBu (1 μg/ml) (EGF + PDBu) for 5 min or thapsigargin (1 μg/ml) (EGF + Thapsigargin) for 60 min at 37°C. EGF (150 ng/ml) was then added for an additional 5 min and the EGF receptors were isolated as in *A*. All samples were run in triplicate. This figure is a composite of representative samples from a single autoradiograph.

To determine whether increases in EGF receptor phosphorylation might be a general mechanism for regulating EGF receptor properties, we examined the state of EGF receptor phosphorylation after treatment of A431 cells with A23187 or thapsigargin. For these experiments, EGF receptors from A431 cells labeled with $^{32}P_i$ were immunoprecipitated with a polyclonal antibody against v-erbB to ensure that receptor purification would not depend on the state of receptor phosphorylation. In cells with control levels of protein kinase C, treatment with 50 μM A23187 for 5 min and 1 μM thapsigargin for 60 min increased EGF receptor phosphorylation approximately 1.5- and 3-fold, respectively, comparable to that obtained after PDBu treatment (Fig. 3 *A* and *C*). The time course and dose response of thapsigargin-induced receptor phosphorylation paralleled that for inhibition of EGF binding (see Fig. 1; data not shown). In protein kinase C-depleted cells, A23187 increased EGF receptor phosphorylation 6-fold and thapsigargin increased EGF receptor phosphorylation 3-fold, whereas PDBu had no significant effect (Fig. 3 *B* and *D*). Analysis of the phosphorylated amino acids indicated that only serine and threonine residues were phosphorylated as a result of these treatments (data not shown). These results suggest that increases in phosphorylation of the EGF receptor may be a

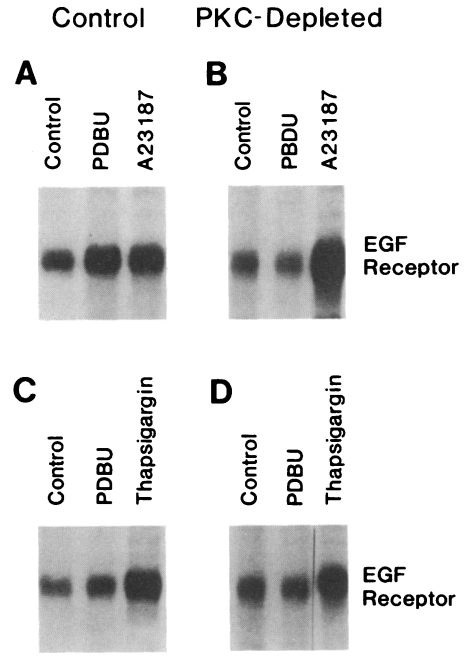


FIG. 3. The calcium ionophore A23187 and thapsigargin stimulate EGF receptor phosphorylation in A431 cells. (*A*) Calcium ionophore treatment of normal cells. Confluent A431 cells were labeled and incubated in the absence (control) or presence of PDBu (1 μg/ml) or A23187 (50 μM) as described in Fig. 2. EGF receptors were isolated by immunoprecipitation with a polyclonal anti-erbB antibody and SDS/PAGE. (*B*) Calcium ionophore treatment of protein kinase C-depleted cells. A431 cells were depleted of protein kinase C and then treated as in *A*. (*C*) Thapsigargin treatment of normal cells. A431 cells were labeled and incubated in the absence (control) or presence of PDBu (1 μg/ml) or thapsigargin (1 μg/ml) as described in Fig. 2. EGF receptors were then isolated as in *A*. (*D*) Thapsigargin treatment of protein kinase C-depleted cells. A431 cells were depleted of protein kinase C and then treated as in *C*. All samples were run in triplicate. This figure is a composite of representative samples from a single autoradiograph. Quantitation of the labeled bands yielded the following: (*i*) normal cells: control, 1678 ± 108 cpm; PDBu, 3040 ± 2 cpm; A23187, 2559 ± 206 cpm; thapsigargin, 4814 ± 356 cpm; (*ii*) protein kinase C-depleted cells: control, 1698 ± 195 cpm; PDBu, 1435 ± 144 cpm; A23187, 10537 ± 3589 cpm; thapsigargin, 3937 ± 630 cpm.

general mechanism for negative regulation of EGF receptor binding and kinase activity.

Several reports have suggested that phosphorylation of one particular residue of the EGF receptor, threonine-654, is critical for modulation of receptor properties via protein kinase C-dependent pathways (7, 8, 10, 13). To determine potential regulatory sites on the EGF receptor that might be involved in modulation of receptor properties by A23187 and thapsigargin, we analyzed tryptic phosphopeptides from EGF receptors isolated from control, A23187, thapsigargin, and PDBu-treated cells. If phosphorylation of threonine-654 is critical for the negative regulation of EGF receptor binding and kinase properties, then EGF receptors isolated from A23187 and thapsigargin-treated cells should also be phosphorylated at this site.

Threonine-654 does appear to be phosphorylated when A431 cells are treated with the calcium ionophore A23187. For the isolation of phosphotryptic peptides, ^{32}P-labeled EGF receptors were immunoprecipitated with anti-erbB antiserum and digested with trypsin, and the tryptic digests were resolved by HPLC on a C18 reverse-phase column in a gradient of 0–50% acetonitrile over a period of 80 min at a flow rate of 1.1 ml/min. The first peak that eluted from the column was inorganic phosphate, as determined by thin-layer electrophoresis (data not shown). Under all treatment conditions, the major peak of radioactivity eluted with a retention time of 35 min, corresponding to ≈22% acetonitrile (Fig. 4*A Left*). Treatment of cells with PDBu (Fig. 4*B Left*) resulted in the appearance of a broad peak or doublet eluting at 14–16 min (9–10% acetonitrile) that has been identified as threonine-654 phosphotryptic peptides (ref. 22; data not shown). A similar peak was seen after treatment of A431 cells with the calcium ionophore A23187 (Fig. 4*C Left*), indicating that threonine-654 is phosphorylated under these conditions. To assess the protein kinase C dependence of the threonine-654 phosphorylation after A23187 treatment, similar studies were performed in protein kinase C-depleted cells. Under our conditions, EGF receptors isolated from cells lacking protein kinase C that had been rechallenged with PDBu do not contain phosphorylated threonine-654, consistent with the role of protein kinase C in mediating this effect (Fig. 4 *A* and *B Right*). In contrast, phosphorylated peptides eluting at the position of threonine-654 are still observed after A23187 treatment of protein kinase C-depleted cells, although the percent of total phosphorylation represented by threonine-654 is greatly reduced relative to cells that contain normal levels of protein kinase C (Fig. 4*C Right*). The relative stoichiometry of EGF receptor phosphorylation at threonine-654 resulting from calcium ionophore treatment is similar to that reported for PDGF treatment of protein kinase C-deficient human fibroblasts (22) and may reflect activation of a similar protein kinase C-independent pathway by both PDGF and calcium ionophore. These results indicate that both PDBu and the calcium ionophore stimulate phosphorylation of threonine-654 under conditions where they effect changes in EGF receptor binding and EGF-stimulated receptor kinase properties.

In contrast, thapsigargin-stimulated phosphorylation of the the EGF receptor occurs at sites other than threonine-654. The elution profiles of EGF receptor phosphopeptides from cells treated with thapsigargin did not contain peaks eluting at the position of threonine-654 (Fig. 5). Similar phosphotryptic peptide maps were obtained after thapsigargin treatment of both protein kinase C-containing and protein kinase C-depleted A431 cells (Fig. 5). Our results raise the possibility that other region(s) of the EGF receptor, when phosphorylated, negatively regulate high-affinity EGF receptor binding and EGF-stimulated tyrosine phosphorylation in intact cells. One potential site for regulation is a neighboring residue, threonine-669, that we have recently identified as the major target of thapsigargin-induced phosphorylation of the EGF receptor (28).

The signalling pathway by which thapsigargin modulates EGF receptor properties is not presently known. Thapsigargin, a hexaoxygenated tetraacylated sesquiterpene lactone, is a natural plant product that was purified from the roots of *Thapsia garganica* L. (Apiaceae) (17, 23). It is a natural skin irritant that has a polyoxygenated terpene structure similar to that of the phorbol ester tumor promoter PMA. Like PMA, thapsigargin promotes and increases histidine decarboxylase

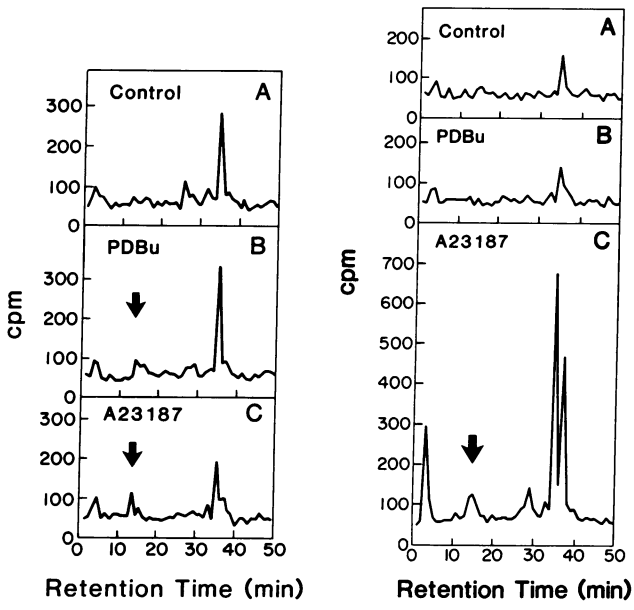


FIG. 4. HPLC phosphotryptic peptide profiles of EGF receptors isolated from control (*Left*) or protein kinase C-depleted (*Right*) A431 cells that had been treated with control medium (*A*), PDBu (*B*), or calcium ionophore A23187 (*C*). EGF receptors were treated and isolated from A431 cells as described in Fig. 3 and then trypsinized and separated by HPLC as described in *Materials and Methods*. These results are representative of at least three independent experiments. Arrows refer to the position of phosphorylated threonine-654.

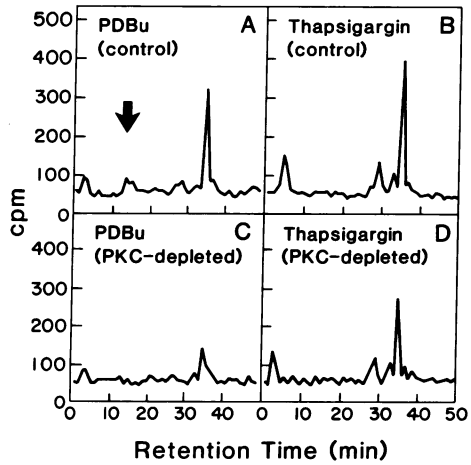


FIG. 5. HPLC phosphotryptic peptide profiles of EGF receptors isolated from control (*A* and *B*) or protein kinase C (PKC)-depleted (*C* and *D*) A431 cells that had been treated with PDBu (*A* and *C*) or thapsigargin (*B* and *D*). EGF receptors were treated and isolated from A431 cells as described in Fig. 3 and analyzed as in Fig. 4. These results are representative of at least six independent experiments with up to 30,000 cpm applied to the HPLC column. Arrow refers to the position of phosphorylated threonine-654.

activity in the skin of mice that have been painted with a subthreshold dose of carcinogen (18). However, thapsigargin does not induce other PMA-like biological responses, such as ornithine decarboxylase production or adhesion of human promyelocytic leukemia (HL-60) cells (18). Thapsigargin has been found to increase intracellular calcium concentrations through calcium influx in platelets (24) and in a neural cell line through an inositol phospholipid-independent mechanism (25). However, the action of thapsigargin appears to differ from that of a calcium ionophore. For example, thapsigargin does not induce K^+ efflux from erythrocytes (26) or histamine release from guinea pig mast cell granules (27). Independent of the particular mechanism, it appears clear from our results that thapsigargin treatment leads to activation of a kinase that can phosphorylate the EGF receptor.

Our results indicate that phosphorylation of residue threonine-654 is not required for negative regulation of EGF receptor binding and kinase activities by thapsigargin. Using agents that activate alternative pathways for down-modulating EGF receptor properties, we show that receptor phosphorylation by serine- and/or threonine-specific kinases appears to be a general regulatory mechanism. Recent work from our laboratory using staurosporine, a protein kinase inhibitor, indicates that the number of high-affinity EGF receptors is negatively regulated in normal untreated cells and that increased high-affinity binding correlates with decreased EGF receptor phosphorylation (unpublished data). Since receptors in untreated cells do not contain phosphorylated threonine-654, these results suggest that phosphorylation at other sites can inhibit high-affinity EGF receptor binding and are in agreement with recent site-directed mutagenesis studies (11, 12). Our present work with thapsigargin supports and extends this conclusion by providing evidence that EGF-stimulated receptor autophosphorylation as well as EGF binding can be regulated independent of threonine-654 phosphorylation. In sum, these results suggest that alternative phosphorylation sites on the EGF receptor may play a regulatory role in normal cells.

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