Phosphorylation of the Epidermal Growth Factor Receptor at Threonine 654 Inhibits Ligand-induced Internalization and Down-regulation*


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To assess the functional significance of phosphorylation of the epidermal growth factor (EGF) receptor at Thr654, we compared the effects of 12-O-tetradecanoyl-13-acetate (TPA) on ligand-induced internalization and down-regulation between wild-type and mutant receptors that contain an alanine substitution at position 654. Activation of protein kinase C with TPA blocked EGF-induced internalization and down-regulation of Thr654 receptors and inhibited in vivo tyrosine kinase activity by 80%. TPA did not inhibit internalization or constitutive EGF receptor internalization, suggesting that protein kinase C activation inhibits only the ligand-induced process. Inhibition by TPA of induced internalization, down-regulation, and kinase activity required threonine at position 654 since full-length Ala654 EGF receptors were significantly resistant to TPA inhibition of these ligand-induced activities. However, C'-terminal truncation further enhanced this resistance to TPA inhibition. The EGF-dependent internalization of kinase-inactive receptors truncated at residue 1022 was also impaired by TPA in Thr654 receptors, but not in Ala654 receptors, indicating that phosphorylation at Thr654 also interferes with tyrosine kinase-independent receptor activities. We conclude that the dominant regulatory effect of protein kinase C on the EGF receptor is mediated through phosphorylation at Thr654 which effectively inactivates the receptor. The submembrane region of the EGF receptor appears to regulate transmission of conformational information from the extracellular ligand-binding site to the cytoplasmic kinase and regulatory domains.

The epidermal growth factor (EGF) receptor is an extensively regulated protein-tyrosine kinase that can modulate multiple cellular processes (1). The magnitude of any single biological response induced by receptor activation depends on ligand-, receptor-, and cell-specific regulatory events. The most important regulator of EGF receptor activity is ligand binding. Occupancy of the single extracellular binding site of each receptor (2) leads to signal transduction across the cytoplasmic membrane and results in a 2-10-fold increase in activity of the cytoplasmic protein-tyrosine kinase (3-5) as well as a 5-10-fold increase in the rate of receptor internalization (6-8) and a 5-50-fold down-regulation of surface receptors (6, 9).

EGF receptor activity is also regulated by covalent phosphorylation/dephosphorylation at specific cytoplasmic sites (10-12). Autophosphorylation of tyrosine residues located in the C'-terminus leads to increased protein-tyrosine kinase activity by release of competitive inhibition of substrate binding (13). The EGF receptor is also an important substrate for the Ca2+/phospholipid-dependent kinase, protein kinase C. Activation of protein kinase C, either by the pharmacological action of phorbol esters (14, 15) or by phosphatidylinositol turnover induced by hormones (16, 17), leads to decreased EGF receptor kinase activity (11, 12). Protein kinase C activity also decreases receptor affinity for its ligand (18-20) and attenuates EGF-induced biological responses (21-24).

The EGF receptor is phosphorylated in vivo at several serine and threonine residues (5, 11, 25). The major in vivo site of phosphorylation by protein kinase C is at Thr654, located 9 residues inside the lipid bilayer and proximal to the consensus tyrosine kinase domain (26, 27). Although substitution of Thr654 with another amino acid prevents some phorbol ester effects on EGF receptor function, other effects are not altered by the substitution (23, 24, 28, 29). There is also considerable variability between experimental systems and cell types in the effects of phorbol esters on both wild-type receptors and receptors mutated at residue 654 with respect to ligand affinity (23, 24, 29, 30), ligand-induced receptor tyrosine kinase activity (23, 24, 29), receptor internalization, down-regulation (20, 21, 29-31), and the mitogenic effect of EGF (21, 24, 32, 33). In addition, a mutation remote to Thr654 has been reported to alter the effects of phorbol esters on EGF receptor function (24). Thus, the functional role of phosphorylation at Thr654 in regulating EGF receptor activity is incompletely defined.

The tyrosine kinase activity of the EGF receptor is the only biochemical property intrinsic to the receptor itself that does not require molecular interaction with other cellular proteins. Ligand-induced enzymatic activity is readily demonstrated in...
isolated receptor preparations (12). Ligand-induced internalization also behaves as an intrinsic property of the receptor even though endocytosis requires receptor interaction with the endocytic apparatus. Human EGF receptors expressed in cells lacking endogenous receptors undergo efficient ligand-dependent internalization (34-36). Importantly, there is a one-to-one stoichiometric relationship between receptor occupancy and both kinase activity and induced internalization that is not necessarily true of other induced responses. Receptor tyrosine kinase activity and induced internalization are thus specific, quantitative indicators of EGF receptor function that are relatively independent of either concurrent or subsequent cellular events that may be regulated by protein kinase C.

In this study, we describe the effects of phorbol esters on in vivo tyrosine kinase activity and induced internalization of a series of mutated human EGF receptors in which alanine is substituted for threonine at position 654. Phorbol ester dramatically decreased both ligand-induced internalization and in vivo receptor tyrosine kinase activity. The major determinant of these effects is phosphorylation at Thr<sup>654</sup>, indicating that this region of the EGF receptor fulfills a critical function in transmembrane signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse EGF was purified from submaxillary glands according to the method of Savage and Cohen (37). Human transferrin (Calbiochem) was iron-loaded as described (38). EGF and transferrin were iodinated using IODO-BEADS (Pierce Chemical Co.) according to the manufacturer's recommendations, and free i<sup>125</sup>I was separated from the radiolabeled ligands by dialysis or by passing the mixture over a 0.8 x 20-cm column of Sephadex G-10 equilibrated with phosphate buffered saline, 12-O-Tetradecanoylphorbol-13-acetate (TPA) (Sigma) was stored as a stock solution in dimethyl sulfoxide or ethanol. Control experiments were performed using solvent alone.

**Construction and Expression of Mutant Human EGF Receptors**—The chimeric expression plasmid pXER places the EGF receptor under control of the SV40 early promoter/enhancer and uses the SV40 late splice/polyadenylation site (39). A mutant dihydroxyproline reductase gene in the opposite orientation provided a dominant selectable marker (40). C'-terminal truncations were prepared in the wild-type EGF receptor containing Thr at residue 654 and in AlaG<sub>b</sub>4. All mutant receptors were transfected into mouse B82 cells, which lack endogenous EGF receptor mRNA and protein; and permanent clonal transfecants were selected using increasing concentrations of methotrexate from 400 nM to 5 μM. At least two independent transfections were used to select each cell line expressing mutant EGF receptors. B2<sub>0</sub> cells were grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Inc.) containing 10% calf serum. A31 cells were obtained from Dr. Harry Haigler (University of California, Irvine) and grown in DMEM containing 10% calf serum. Human foreskin fibroblasts were prepared and cultured as described (8, 41).

**Measurement of Specific Internalization Rates**—Cells grown to confluence in 35-mm dishes were switched from DMEM plus 10% calf serum to serum-free DMEM containing 20 mM HEPES (pH 7.4) and 0.1% bovine serum albumin and no bicarbonate 18 h before experiments. Binding experiments were initiated by adding <sup>125</sup>I-labeled ligand at 37 °C. At 1-min intervals, plates were shifted to 0 °C and rapidly rinsed six times with 2 ml of ice-cold buffer. The amounts of ligand associated with the surface and interior of the cells were determined by solubilizing (41) using 50 mM glycine HCl, 100 mM NaCl, 2 mg/ml polyvinylpyrrolidone, 2 μM urea (pH 3.0). Nonspecific binding was determined in the presence of at least a 200-fold molar excess of unlabeled EGF or transferrin and was measured by subtracting binding to the EGF receptor-negative parental B82 cell line. Nonspecific binding was generally less than 5% of total binding. Values for surface-bound and internalized ligand were corrected for nonspecific binding and for spill-over from the interior and surface of the cell, respectively. The data were analyzed using internalization plots by converting the amount of surface-associated ligand at each point to the integral of surface binding (from zero time to the time point) and plotting this against the amount of internalized ligand (42, 43). The specific internalization rate (k) of the receptors, which is directly proportional to the slope of the plots, was determined by linear regression. Correlation coefficients for these plots were >0.98.

**RESULTS**

**TPA Prevents Ligand-induced Internalization of EGF Receptors**—The effect of TPA on EGF receptor internalization was investigated using mouse B2<sub>0</sub> cells that express human EGF receptors (29, 35, 36). Cells that express wild-type EGF receptors (Thr<sup>654</sup>) were treated for 10 min at 37 °C with 100 nM TPA and then incubated with <sup>125</sup>I-EGF for 1 to 5 min. The k<sub>e</sub> of the EGF receptor was then determined using the internalization plot technique (42, 43). Fig. 1A shows that treatment with TPA decreased the value of k<sub>e</sub> from 0.31 to 0.03.
that TPA increased the rate of transferrin receptor internalization in all cell types examined (Fig. 2B). These results demonstrated that phosphorylation in the inhibition of EGF receptor internalization is the rate at which unoccupied EGF receptors or those lacking a cytoplasmic domain enter the cell. This rate is consistent with random entrapment in endocytic structures (42, 49) and is similar to that reported for mutant "internalization-defective" low density lipoprotein, transferrin, and asialoglycoprotein receptors (50–52). Our data indicate that EGF receptor endocytosis through this nonsaturable, constitutive internalization pathway is not inhibited by TPA.

To ensure that inhibition of ligand-induced EGF receptor internalization by TPA is not unique to B82 cells expressing transfected receptors, we examined both human fibroblasts and A431 cells. As shown in Fig. 2A, TPA reduced the specific internalization rate of EGF receptors in each of these cell types. To determine whether the inhibition of internalization was receptor-specific, the effect of TPA on transferrin receptor internalization was also measured. Endocytosis of the transferrin receptor is independent of receptor occupancy and occurs exclusively by a constitutive process (42, 53). In contrast to its inhibitory effect on EGF receptor internalization, we found that TPA increased the rate of transferrin receptor internalization in all cell types examined (Fig. 2B). These results indicate that TPA selectively inhibits ligand-induced internalization of the EGF receptor.

Ala654 Receptors Are Resistant to TPA Inhibition of Ligand-induced Internalization—To determine the role of Thr654 phosphorylation in the inhibition of EGF receptor internalization, the effect of TPA on the internalization of Ala654 receptors was measured. As shown in Fig. 3, EGF-dependent endocytosis of Ala654 receptors was resistant to inhibition by TPA. Although TPA reduced the specific internalization rate of these receptors from 0.44 to 0.23 min⁻¹ (Fig. 3), it failed to decrease the kₙ, to the low values typical of constitutive endocytosis (0.03 min⁻¹), as was observed with Thr654 receptors. Endocytosis of Ala654 EGF receptors expressed in other cell types was also resistant to inhibition by TPA. The kₙ values for Ala654 EGF receptors expressed in 3T3 cells were 0.19 and 0.15 min⁻¹ in the absence and presence of TPA, respectively (data not shown). Although they are resistant to complete inhibition of induced internalization by TPA, full length Ala654 EGF receptors do retain partial sensitivity.

TPA Inhibition of Ligand-induced Internalization Is Independent of Receptor Tyrosine Kinase Activity—EGF receptors truncated at residue 1022 are internalized in a ligand-induced manner through a nonsaturable, low affinity endocytic pathway kinetically distinct from the normal induced pathway (36). Kinase-active c’1022 receptors are internalized through both pathways, whereas kinase-inactive Met1022 c’1022 receptors utilize the low affinity pathway exclusively. Endocytosis through the low affinity pathway results in receptor down-regulation, but kinase-inactive Met1022 c’1022 receptors do not exhibit the same levels of down-regulation due to their reduced internalization rate.

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generate any other biological response (36). To determine whether TPA affects induced internalization of kinase inactive EGF receptors, the specific internalization rates of kinase-active and inactive c't022 receptors were measured in the absence and presence of TPA. We found that TPA dramatically inhibited internalization of both c't022 and Metc'1022 receptors (Fig. 4). The specific internalization rate of each receptor in the presence of TPA was between 0.01 and 0.05 min⁻¹, values consistent with constitutive internalization. In contrast, Ala₅₆₄ c't022 truncated receptors were highly resistant to the inhibitory effect of TPA (Fig. 4, C and D), demonstrating that TPA inhibits ligand-induced internalization of EGF receptors by a mechanism dependent on Thr₆₅₄ phosphorylation but independent of intrinsic receptor tyrosine kinase activity. Removal of the C'-terminal 164 amino acids did not change the strong inhibitory effect of TPA on Thr₆₅₄ EGF receptors (Fig. 1A versus Fig. 4A), but largely abolished the residual effect of TPA on Ala₅₆₄ EGF receptors (Fig. 3 versus Fig. 4C). This result suggests that, although Thr₆₅₄ is the major site at which TPA interferes with induced internalization, secondary effects are apparently dictated by the C' terminus.

Because the slopes of internalization plots are not sensitive to ligand binding affinity (42, 43), decreased kᵢ values cannot be attributed to decreased EGF receptor affinity mediated by TPA. Phorbol ester effects on EGF receptor affinity have been reported to be independent of phosphorylation at Thr₆₅₄ (23, 24). In B82 cells, TPA decreased the affinity of both Thr₆₅₄ and Ala₅₆₄ EGF receptors, but the effect on Ala₅₆₄ receptor affinity was highly variable and less pronounced than on Thr₆₅₄ receptors (Ref. 59 and data not shown). Furthermore, TPA did not alter the affinity of either Thr₆₅₄ or Ala₅₆₄ C'-terminal truncated receptors in either kinase-active or inactive constructions (data not shown). Changes in receptor affinity thus do not correlate with TPA-mediated inhibition of endocytosis.

**TPA Prevents Ligand-induced Down-regulation of EGF Receptors—Down-regulation of the EGF receptor results from induced internalization followed by receptor degradation (6, 9). To investigate the effect of TPA on receptor down-regulation, cells expressing Thr₆₅₄ or Ala₅₆₄ EGF receptors were treated with EGF in the absence and presence of TPA. Residual cell-surface EGF receptors were then measured by ¹²⁵I-EGF-binding. As shown in Fig. 5A, B82 cells down-regulate full-length Thr₆₅₄ EGF receptors to ~30% of the control level within 1 h of exposure to EGF. TPA alone induced a 20% reduction in the number of surface receptors, but prevented ligand-induced down-regulation of Thr₆₅₄ EGF receptors (Fig. 5A). EGF also reduced the surface density of Ala₅₆₄ receptors to ~30% of the initial values, indicating that this substitution does not prevent receptor down-regulation (Fig. 5B). In contrast to its inhibitory effect on Thr₆₅₄ receptor down-regulation, TPA did not affect the ability of EGF to stimulate Ala₅₆₄ receptor down-regulation (Fig. 5B). TPA impaired EGF-induced down-regulation of Thr₆₅₄ c't022 truncated receptors (Fig. 5C), but not of Ala₅₆₄ c't022 truncated receptors (Fig. 5D). These results were confirmed by measuring surface receptor number by flow cytometry using an anti-EGF receptor monoclonal antibody and by immunoprecipitation of ¹²⁵I-labeled surface receptors (data not shown). Thus, decreased ligand binding reflects a loss of receptor mass from the cell surface.

The role of Thr₆₅₄ in phorbol ester-mediated inhibition of EGF receptor internalization and down-regulation was further investigated by determining the cellular distribution of receptors in the presence of TPA. B82 cells expressing Thr₆₅₄ or Ala₅₆₄ EGF receptors were treated with EGF, TPA, or both. EGF receptors were then identified by immunofluorescence (35). As shown in Fig. 6, both Thr₆₅₄ and Ala₅₆₄ EGF receptors were uniformly distributed on the cell surface prior to treatment with EGF. Treatment with TPA alone did not alter the staining pattern. Following exposure to EGF, receptors were found predominantly in punctate cytoplasmic clusters, consistent with induced internalization. TPA completely eliminated EGF-induced redistribution of Thr₆₅₄ receptors so that they remained diffusely distributed on the cell surface. Ala₅₆₄

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**Fig. 4. Effects of TPA on induced internalization of kinase-active and inactive c't022 truncated EGF receptors containing Thr₆₅₄ and Ala₅₆₄.** The specific internalization rate of Thr₆₅₄ and Ala₅₆₄ c't022 truncated EGF receptors was determined at a concentration of 0.17 nM ¹²⁵I-EGF without (○) or with (●) prior treatment with 100 nM TPA for 10 min. A, Thr₆₅₄ c't022 truncated kinase-active (kin +) EGF receptors, kᵢ = 0.25 and 0.04 min⁻¹ in the absence and presence of TPA, respectively. B, Thr₆₅₄ Met₇₂ c't022 truncated kinase-inactive (kin -) receptors, kᵢ = 0.11 and 0.02 min⁻¹, respectively. C, Ala₅₆₄ c't022 truncated kinase-active receptors, kᵢ = 0.13 and 0.10 min⁻¹, respectively. D, Ala₅₆₄ Met₇₂ c't022 truncated kinase-inactive receptors, kᵢ = 0.09 and 0.07 min⁻¹, respectively.
EGF Receptor Regulation by Protein Kinase C

EGF receptors, however, were found in cytoplasmic clusters despite TPA treatment and were thus resistant to its inhibitory effect. TPA also failed to inhibit ligand-induced redistribution of Ala<sup>654</sup> c<sup>1022</sup> truncated receptors (data not shown). These data confirm that phosphorylation of the EGF receptor at Thr<sup>654</sup> by protein kinase C is sufficient to block ligand-induced endocytosis and receptor down-regulation.

**Ala<sup>654</sup> Receptors Are Resistant to Inhibition of Kinase Activity by TPA**—Phosphorylation of Thr<sup>654</sup> is reported to decrease EGF-stimulated protein phosphotyrosine content of cells in <i>vivo</i> and to reduce EGF receptor tyrosine kinase activity assayed in <i>vitro</i> (11, 12, 21). We developed a procedure to quantitate EGFR-dependent receptor tyrosine kinase activity in <i>vivo</i> and used this method to compare the effects of TPA on Thr<sup>654</sup> and Ala<sup>654</sup> EGF receptors. TPA decreased EGF-induced tyrosine kinase activity of Thr<sup>654</sup> receptors to 75% of the activity in untreated cells (Fig. 7). In contrast to the large inhibitory effect on Thr<sup>654</sup> receptors, there was less than a 10% decrease in the specific activity of EGF-stimulated self-phosphorylation of Ala<sup>654</sup> receptors after treatment with TPA. Inhibition of Thr<sup>654</sup> receptor kinase activity by TPA could not be overcome by longer exposure to EGF or by increasing the concentration of EGF (data not shown), indicating that inhibition of EGF receptor tyrosine kinase activity in <i>vivo</i> by TPA is not due to reduced receptor mass or reduced receptor affinity for EGF.

Because some effects of protein kinase C on EGF receptor function are reported to be independent of phosphorylation at Thr<sup>654</sup> (23, 24), we measured the effects of TPA on <i>vitro</i> tyrosine kinase activity of Thr<sup>654</sup> and Ala<sup>654</sup> EGF receptors

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**FIG. 5.** Effect of TPA on EGF-induced receptor down-regulation. B82 cells expressing human EGF receptors were treated for 10 min without (O) or with (●) TPA prior to exposure to EGF at 37°C for the indicated times. Bound EGF was removed by exposure to acetic acid and residual EGF receptors were measured by incubating with <i>12</i>II-EGF. A, Thr<sup>654</sup> EGF receptors. WI, wild type; C, control cells treated with TPA only. B, Ala<sup>654</sup> EGF receptors. C, Thr<sup>654</sup> c<sup>1022</sup> EGF receptors. D, Ala<sup>654</sup> c<sup>1022</sup> EGF receptors.

**FIG. 6.** Immunofluorescence microscopy analysis of effects of TPA and EGF on Thr<sup>654</sup> and Ala<sup>654</sup> EGF receptors in B82 cells. The cells were treated without or with 100 nM TPA for 10 min prior to incubation in the absence or presence of 17 nM EGF for 20 min at 37°C. The cells were then fixed and exposed to anti-EGF receptor monoclonal antibody, and immunofluorescence analysis was performed.

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**FIG. 7.** Effect of TPA on tyrosine kinase activity in B82 cells expressing Thr<sup>654</sup> and Ala<sup>654</sup> EGF receptors with progressive C-terminal truncations. The cells were treated for 10 min with the indicated concentrations of TPA, and 10 nM EGF was added 30 s prior to cell extraction. Autoradiographs in A and B show extracts from cells expressing holo Thr<sup>654</sup> and Ala<sup>654</sup> EGF receptors (EGFR), respectively; C shows the activity calculated from A and B. Autoradiographs in D and E show extracts from cells expressing Thr<sup>654</sup> c<sup>1022</sup> and Ala<sup>654</sup> c<sup>1022</sup> EGF receptors, respectively; F shows the activity calculated from D and E. Autoradiographs in G and H show extracts from cells expressing Thr<sup>654</sup> c<sup>973</sup> and Ala<sup>654</sup> c<sup>973</sup> EGF receptors, respectively; I shows activity calculated from G and H. Because the c<sup>973</sup> EGFR receptor does not self-phosphorylate, the activity shown in I is calculated from total cell protein tyrosine phosphorylation per immunostained EGF receptor. The carets show the position of the EGFR as determined from anti-EGF receptor immunostaining. ●, Thr<sup>654</sup> EGF receptors; ■, Ala<sup>654</sup> EGF receptors.
with C'-terminal truncations to residues 1022 and 973. Fig. 7 shows that increasing concentrations of TPA inhibited the tyrosine kinase activity of Thr654 receptors with C'-terminal truncations as efficiently as in cells expressing the full-length receptor. All receptors containing Ala654, however, were resistant to these inhibitory effects. The kinase activity of full-length and Ala654 c'1022 EGF receptors could be partially inhibited by very high concentrations of TPA. Because c'973 EGF receptors lack self-phosphorylation sites (54), densitometric analysis of autoradiographs produced from cells expressing these receptors quantitate in vivo substrate phosphorylation only. The Ala654 c'973 EGF receptors appear completely resistant to the inhibitory effects of TPA and exhibit full kinase activity even at very high TPA concentration. These results indicate that phosphorylation of Thr654 is the major determinant of phorbol ester-induced inhibition of the tyrosine kinase activity of the EGF receptor. As was the case for induced internalization, secondary effects of TPA on EGF receptor kinase activity appear to involve the C' terminus because its removal abolishes residual effects on Ala654 receptors.

**DISCUSSION**

We have shown that activation of protein kinase C by phorbol esters strongly inhibits the two ligand-induced activities that behave as intrinsic properties of the EGF receptor. TPA prevented ligand-induced receptor internalization and inhibited EGF-induced in vivo tyrosine kinase activity. Ligand-induced internalization of EGF receptors through either the high or low affinity endocytic pathway (36, 42) was reduced by TPA to very low levels consistent with constitutive receptor internalization. EGF receptor down-regulation, which results from induced internalization through either pathway, was also prevented by treatment with TPA. Because TPA did not impair constitutive endocytosis of EGF receptors, the effects of protein kinase C on the EGF receptor appear to be on ligand-induced activities.

The ability of TPA to coordinately inhibit both EGF receptor kinase activity and induced internalization contrasts with the effect of C'-terminal truncation on receptor activity. Removal of the C-terminal region distal to residue 973 results in a receptor with enhanced kinase activity, but with a severely compromised ability to undergo induced internalization (36, 54, 55). Despite removal of this large regulatory region, TPA efficiently inhibits in vivo kinase activity of c'973 EGF receptors. Therefore, protein kinase C does not require functional regulatory domains located in the C' terminus distal to the conserved tyrosine kinase domain for its inhibitory action. Because intrinsic tyrosine kinase activity is required for normal ligand-induced internalization of EGF receptors (36, 42), it was possible that inhibition of internalization was secondary to TPA's effect on receptor kinase activity. This is not the case, however, because TPA efficiently inhibited internalization of kinase-inactive Met621 c'1022 receptors. Protein kinase C inhibition of both EGF receptor kinase activity and induced internalization may be secondary to a more proximal molecular event.

In contrast to Thr654 EGF receptors, mutant Ala654 EGF receptors were resistant to the inhibitory effects of TPA on intrinsic receptor functions. The Ala654 substitution conferred TPA resistance to both C'-terminal truncated and full-length receptors. Induced internalization of Ala654 EGF receptors through both high and low affinity endocytic pathways was resistant to inhibition by TPA. Similarly, the ability of TPA to inhibit kinase activity of c'973 receptors was strictly dependent on tyrosine at position 654. In all receptor constructions, the ability of TPA to inhibit EGF receptor activity correlates with the presence of the protein kinase C phosphorylation site at Thr654 regardless of other receptor-specific properties that influence its activity. The submembrane region surrounding Thr654 is ideally situated to play an important role in the signal transduction process since it extends from residue 645 at the distal end of the membrane-spanning region to the proximal end of the consensus kinase domain at residue 694. The submembrane regions of the closely related c-erbB, neu/HER2, and Xmrk gene products all demonstrate extensive sequence homology to this region of the human EGF receptor (56-58), indicating that it serves a critical function. Our results demonstrate that phosphorylation at Thr654 is sufficient to prevent EGF receptor activation by ligand. Phosphorylation at this site could prevent the cytoplasmic portion of the EGF receptor from assuming a stable conformation required for ligand-induced functions. Regardless of the mechanism involved, it is clear that the dominant regulatory effect of protein kinase C on EGF receptor function is mediated by phosphorylation at Thr654.

Although full-length Ala654 EGF receptors were resistant to low concentrations of TPA, high concentrations inhibited both induced internalization and in vivo tyrosine kinase activity by up to 50%. TPA effects which are independent of Thr654 may be due to phosphorylation of the EGF receptor at other serine or threonine residues (54) or may be indirect effects due to phosphorylation of receptor substrates or other regulatory molecules. Alternatively, protein kinase C may form stable, inactive, transition-state complexes with EGF receptors if phosphorylation fails to occur because the Thr654 acceptor site is eliminated by substitution. It is thus possible that the effects of TPA on Ala654 EGF receptor function may not occur to a significant extent in Thr654 receptors. Phorbol ester effects on Ala654 EGF receptors apparently require the presence of the regulatory C'-terminal region because truncated Ala654 receptors are virtually unaffected by TPA. However, studies by Countaway et al. (59) indicate that alternative use of the Thr654, Ser1046, and Ser1047 phosphorylation sites does not alter phorbol ester responses. Therefore, removal of the Ser1046 and Ser1047 phosphorylation sites could not explain the differing effects seen with holo and C'-terminal truncated Ala654 EGF receptors.

Activation of protein kinase C reduces EGF receptor affinity as well as ligand-induced tyrosine kinase activity and receptor internalization. This not only blocks the biological activity of EGF, but also prevents EGF refractory cells from utilizing the growth factor. The receptor would thus become functionally invisible. A wide variety of physiological agents stimulate hydrolysis of phosphatidylinositol by phospholipase C (60, 61), which leads to diacylglycerol production and activation of protein kinase C (62, 63). Phospholipase C-γ is phosphorylated on tyrosine in vivo and in vitro by the EGF receptor (64-66), and EGF stimulates in vivo phospholipase C activity and activation of protein kinase C (67, 68). Homologous and heterologous regulation of EGF receptor activity may thus be mediated in part by protein kinase C. Activation of protein kinase C occurs rapidly in vivo (60, 61, 69) and leads to inhibition of EGF receptor kinase activity and endocytosis within seconds to minutes of stimulation (data not shown). Recovery from the inhibitory effects of protein kinase C is also a relatively rapid process. Rapid metabolism of diacylglycerol in vivo (61, 69), down-regulation of protein kinase C activity (70), and dephosphorylation of its substrates by specific phosphatases (71) potentially allow recovery of a fully responsive EGF receptor system from protein kinase C mediated inhibition. Recovery from TPA may be slower than...
recovery from diacylglycerol due to the slow metabolism of TPA. However, endocytic down-regulation of EGF receptors occurs much more slowly (Fig. 5) than the protein kinase C-mediated process. In addition, because of the necessity of de novo receptor synthesis, full recovery from endocytotic down-regulation requires several hours. Regulation of the EGF receptor by protein kinase C thus represents a rapid, flexible system for coupling heterologous stimuli to the signal transduction pathway.

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