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


From the Department of Pathology, Division of Cell Biology and Immunology, and the **Veterans Administration Medical Center and Division of Hematology and Oncology, University of Utah Medical School, Salt Lake City, Utah 84132 and the **Department of Medicine, Division of Endocrinology and Metabolism, and The Center for Molecular Genetics and the **Howard Hughes Medical Institute, University of California at San Diego, La Jolla, California 92039

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 transmission 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 Ca'/?/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 inefficient ligand-dependent internalization (34-36). Importantly, there is a one-to-one stoichiometric relationship between receptor occupancy and both kinase activity and internalized ligand 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(654), 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 [125]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 dihydrofolate 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 AlaGb4 mutant EGF receptors (29) as described (39). Similar mutations were placed in EGF receptor cDNA mutated to abolish receptor tyrosine kinase activity by substituting Met for Lys at residue 721 (39). All mutant sequences were confirmed. cDNAs were transfected into mouse B82 cells, which lack endogenous EGF receptor mRNA and protein; and permanent clonal transfectants were selected using increasing concentrations of methotrexate from 400 \( \mu M \) to 5 \( \mu M \). At least two independent transfections were used to select each cell line expressing mutant EGF receptors. B92 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Inc.) containing 10% calf serum. A431 cells were obtained from Dr. Harry H€ager (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 confluency 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 [125]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 acid stripping (41) using 50 mM glycine, HCl, 100 mM NaCl, 2 mg/ml polyclonal mouse anti-EGF or transferrin or by measuring binding to the EGF receptor-negative parental B82 cell line. Nonspecific binding was generally <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.

**EGF Receptor Down-regulation**—Cells expressing normal or mutant EGF receptors were plated at 2 x 10^5 cells/well in 12-well plates. 24 h later, the cells were treated with or without 100 nM TPA for 8 min at room temperature. The medium was then removed, and the cells were washed twice with binding buffer containing binding buffer. The cells then were transferred to polyvinylidene difluoride membranes (Immobilon) and were blocked with 2.5% bovine serum albumin and 0.1% NaN₃ in 10 mM Tris-HCl (pH 7.5), 0.9% NaCl, and 0.05% Tween 20 (TBS-Tween) for 2 h at room temperature, followed by incubation with 125I-labeled mouse monoclonal anti-phosphotyrosine (PY 20) (35) and rabbit polyclonal anti-EGF receptor (R1) (47) antibodies for 2 h at room temperature. The membranes were washed in TBS-Tween and immunostained by incubation with goat anti-rabbit IgG–alkaline phosphatase conjugate using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

**Densitometry of Autoradiographs**—The 125I-labeled mouse monoclonal anti-phosphotyrosine (PY 20) (35) and rabbit polyclonal anti-EGF receptor (R1) (47) antibodies for 2 h at room temperature. The membranes were washed in TBS-Tween and immunostained by incubation with goat anti-rabbit IgG–alkaline phosphatase conjugate using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the substrate.

**RESULTS**

**TPA Prevents Ligand-induced Internalization of EGF Receptors**—The effect of TPA on EGF receptor internalization was investigated using mouse B92 cells that express human EGF receptors (29, 35, 36). Cells that express wild-type EGF receptors (Thr(654)) were treated for 10 min at 37°C with 10 nM TPA and then incubated with [125I]EGF for 1-5 min. The k, 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, from 0.91 to 0.03.
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 or down-regulation (35, 36, 39, 42).

High concentrations of EGF are inhibitory to receptor internalization in cells expressing high receptor numbers due to saturation of the endocytic apparatus (36, 42, 48). In B82 cells that express ~150,000 ThrGs4 EGF receptors/cell, the $k_i$ was decreased from 0.31 min$^{-1}$ at an $^{125}$I-EGF concentration of 0.17 nM to 0.06 min$^{-1}$ at 18.3 nM (Fig. 1, A versus B). TPA had a relatively small inhibitory effect on EGF receptor internalization at high levels of receptor occupancy (Fig. 1B). The $k_i$ in the presence of TPA at both high and low levels of receptor occupancy was 0.03 min$^{-1}$, which is the same as constitutive EGF receptor internalization. Constitutive internalization is the rate at which unoccupied RGF receptors or those lacking a cytoplasmic domain enter the cell.2 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 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 ThrGs4 phosphorylation in the inhibition of EGF receptor internalization, the effect of TPA on 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 $k_i$ to the low values typical of constitutive endocytosis (0.03 min$^{-1}$), as was observed with ThrGs4 receptors. Endocytosis of Ala654 EGF receptors expressed in other cell types was also resistant to inhibition by TPA. The $k_i$ 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 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 c1022 receptors are internalized through both pathways, whereas kinase-inactive Met1022 c1022 receptors utilize the low affinity pathway exclusively. Endocytosis through the low affinity pathway results in receptor down-regulation, but kinase-inactive Met1022 c1022 receptors do not

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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'1022 receptors were measured in the absence and presence of TPA. We found that TPA dramatically inhibited internalization of both c'1022 and Met721 c'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, Ala654 c'1022 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 Ala654 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 not been reported to be independent of phosphorylation at Thr654 (23, 24). In B82 cells, TPA decreased the affinity of both Thr654 and Ala654 EGF receptors, but the effect on Ala654 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 Ala654 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 Ala654 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 Ala654 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 Ala654 receptor down-regulation (Fig. 5B). TPA impaired EGF-induced down-regulation of Thr654 c'1022 truncated receptors (Fig. 5C), but not of Ala654 c'1022 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 Ala654 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 Ala654 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. Ala654...

**FIG. 4. Effects of TPA on induced internalization of kinase-active and inactive c'1022 truncated EGF receptors containing Thr654 and Ala654.** The specific internalization rate of Thr654 and Ala654 c'1022 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'1022 truncated kinase-active (kin +) EGF receptors, kᵢ = 0.25 and 0.04 min⁻¹ in the absence and presence of TPA, respectively. B, Thr654 Met721 c'1022 truncated kinase-inactive (kin -) receptors, kᵢ = 0.11 and 0.02 min⁻¹, respectively. C, Ala654 c'1022 truncated kinase-active receptors, kᵢ = 0.13 and 0.10 min⁻¹, respectively. D, Ala654 Met721 c'1022 truncated kinase-inactive receptors, kᵢ = 0.09 and 0.07 min⁻¹, respectively.
EGF Receptor Regulation by Protein Kinase C

FIG. 5. Effect of TPA on EGF-induced receptor down-regulation. B82 cells expressing human EGF receptors were treated for 10 min without (○) 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 125I-EGF. A, Thr654 EGF receptors. WT, wild type; □, control cells treated with TPA only. B, Ala654 EGF receptors. C, Thr654 c'1022 EGF receptors. D, Ala654 c'1022 EGF receptors.

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 Ala654 c'1022 truncated receptors (data not shown). These data confirm that phosphorylation of the EGF receptor at Thr654 by protein kinase C is sufficient to block ligand-induced endocytosis and receptor down-regulation.

Ala654 Receptors Are Resistant to Inhibition of Kinase Activity by TPA—Phosphorylation of Thr654 is reported to decrease EGF-stimulated protein phosphotyrosine content of cells in vivo and to reduce EGF receptor tyrosine kinase activity assayed in vitro (11, 12, 21). We developed a procedure to quantitate EGF-dependent receptor tyrosine kinase activity in vivo and used this method to compare the effects of TPA on Thr654 and Ala654 EGF receptors. TPA decreased EGF-induced tyrosine kinase activity of Thr654 receptors to 75% of the activity in untreated cells (Fig. 7). In contrast to the large inhibitory effect on Thr654 receptors, there was less than a 10% decrease in the specific activity of EGF-stimulated self-phosphorylation of Ala654 receptors after treatment with TPA. Inhibition of Thr654 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 vivo 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 Thr654 (23, 24), we measured the effects of TPA on in vivo tyrosine kinase activity of Thr654 and Ala654 EGF receptors.

FIG. 6. Immunofluorescence microscopy analysis of effects of TPA and EGF on Thr654 and Ala654 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.

EGF TPA

Thr654

Ala654

- -

+ -

- +

+ +

FIG. 7. Effect of TPA on tyrosine kinase activity in B82 cells expressing Thr654 and Ala654 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 Thr654 and Ala654 EGF receptors (EGFR), respectively; C shows the activity calculated from A and B. Autoradiographs in D and E show extracts from cells expressing Thr654 c'1022 and Ala654 c'1022 EGF receptors, respectively; F show the activity calculated from D and E. Autoradiographs in G and H show extracts from cells expressing Thr654 c'973 and Ala654 c'973 EGF receptors, respectively; I shows activity calculated from G and H. Because the c'973 EGF 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 EGF receptor as determined from anti-EGF receptor immunostaining. ●, Thr654 EGF receptors; ■, Ala654 EGF receptors.
with C' terminus trunctations to residues 1022 and 973. Fig. 7 shows that increasing concentrations of TPA inhibited the tyrosine kinase activity of Thr$^{654}$ receptors with C'-terminal truncations as efficiently as in cells expressing the full-length receptor. All receptors containing Ala$^{654}$, however, were resistant to these inhibitory effects. The kinase activity of full-length and Ala$^{654}$ 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 Ala$^{654}$ 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 Thr$^{654}$ is the major determinant of phorbol ester-inhibtion 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 Ala$^{654}$ 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 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 of the EGF receptor (60, 61), which leads to diacylglycerol production and activation of protein kinase C (62, 63). Phospholipase C-γ is phosphorylated 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 with intact substrates (72).
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 endocytic down-regulation requires several hours. Regulation of the EGF receptor by protein kinase C thus represents a rapid, flexible system for controlling heterologous stimuli to the signal transduction pathway.

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