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Regulation of the Transmodulated Epidermal Growth Factor Receptor by Cholera Toxin and the Protein Phosphatase Inhibitor Okadaic Acid

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Abstract Addition of tumor promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), to many cell lines results in a decrease of ^{125}I-epidermal growth factor (EGF) binding and increased serine/threonine phosphorylation of the EGF receptor in a process termed transmodulation. It is, however, unclear whether or not receptor phosphorylation is causally related to the inhibition of high affinity EGF binding. We have investigated the significance of phosphorylation/dephosphorylation events in the mechanism of PMA-induced transmodulation using the adenylate cyclase activator cholera toxin and the serine/threonine protein phosphatase inhibitor okadaic acid. In Rat-1 fibroblasts treated at 37°C, PMA induced a rapid decrease in EGF binding which persisted for 3 hours. In contrast, cells exposed to PMA in the presence of cholera toxin exhibited a marked recovery of binding within 60 minutes. The PMA-stimulated decrease in binding correlated with a rapid increase in the phosphorylation state of the EGF receptor. While phosphorylation of the receptor was sustained at an elevated level for at least three hours in cells receiving PMA alone, EGF receptor phosphorylation decreased between 1 and 3 hours in cells treated with PMA and cholera toxin. Furthermore, the cholera toxin-stimulated return of EGF binding was inhibited by treatment with the phosphatase inhibitor okadaic acid. These results suggest that a cholera toxin-activated phosphatase can increase binding capacity of the transmodulated EGF receptor in Rat-1 cells. Cholera toxin treatment elicited a qualitatively similar response in cells transmodulated by platelet-derived growth factor (PDGF). Okadaic acid antagonized the natural return of binding observed in cells stimulated with PDGF alone, indicating that a dephosphorylation event may be required for the recovery of normal EGF binding after receptor transmodulation.

Key words: phorbol ester, phosphorylation, epidermal growth factor binding, platelet-derived growth factor

The epidermal growth factor (EGF) receptor is a 170 kD transmembrane tyrosine kinase. Binding of the peptide ligand, EGF, to the extracellular portion of the receptor stimulates the tyrosine kinase activity of the receptor cytoplasmic domain (Ushiro and Cohen, 1980). Activation of the receptor kinase and the resulting tyrosine phosphorylation of various cellular proteins are primary events in a mitogenic cascade of responses which cumulatively lead to cell division (Honegger et al., 1987; Chen et al., 1987). Ultimately the growth stimulatory signal is attenuated after internalization and lysosomal degradation of the receptor-ligand complex (Schlessinger et al., 1978; Haigler et al., 1979).

In many cell lines, other serum-derived growth factors act coordinately with EGF to stimulate cell cycle traverse and the initiation of DNA synthesis (Leof et al., 1982; Wharton et al., 1983). Similarly, tumor promoting phorbol esters have been shown to act synergistically with EGF to elicit a mitogenic response in some fibroblasts (Dicker and Rozengurt, 1978; Frantz et al., 1979). Communication at the receptor level may provide the cell with a means by which to regulate the physiological effects of such growth modulating agents. Stimulation of intact fibroblasts with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), results in the phosphorylation of the EGF receptor at multiple sites through activation of the calcium/phospholipid dependent protein kinase C (Cochet et al., 1984; Iwashita and Fox, 1984; Davis and Czech, 1984). Furthermore, treatment of cells with phorbol ester results in the inhibition of ^{125}I-EGF high affinity binding in a process termed transmodulation (Lee and Weinstein, 1978; Shoyab et al., 1979). Addition of activated protein kinase C to isolated cell membranes or solubilized, purified EGF receptor in vitro leads

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to a reconstitution of the binding inhibition seen in intact cells, as well as to an increase in EGF receptor phosphorylation (Downward et al., 1985; Fearn and King, 1985). Because of the correlation between phorbol ester-induced phosphorylation and the reduction of EGF receptor binding capacity, it has been postulated that phosphorylation of the receptor is directly responsible for transmodulation of EGF receptor affinity. However, the site(s) of phosphorylation which may mediate this transmodulation have not yet been identified, and the exact mechanism of action by which PMA regulates EGF binding remains unclear (Countaway et al., 1990).

Several lines of evidence suggest that the protein kinase modulating agent cyclic AMP may influence the transmodulation pathway. Catecholamines which elevate intracellular cyclic AMP levels in rat adipocytes decrease radiolabeled EGF binding by approximately 50% (Pessin et al., 1983). Additionally, cholera toxin, a potent activator of adenylate cyclase (O'Keefe and Cuatrecasas, 1974), potentiates the phorbol ester-induced reduction of EGF binding in Balb/c-3T3 mouse fibroblasts, although it has no significant effect on binding when added to cells alone (Olashaw et al., 1986). However, increasing the concentration of cyclic AMP in the mouse epidermal cell line HEL-37 dramatically inhibits protein kinase C-induced transmodulation (Gainer and Murray, 1986). These opposing effects of intracellular cyclic AMP in distinct mouse cell lines suggest that heterologous regulation of the EGF receptor by phorbol ester may occur through cell type specific pathways.

The purpose of these experiments is to elucidate the mechanism by which cyclic AMP influences the phorbol ester regulation of EGF receptor binding capacity in Rat-1 fibroblasts and to evaluate the relevance of phosphorylation/dephosphorylation events in this pathway. We report here that the cyclic AMP elevating agent cholera toxin enhances the recovery of EGF binding after PMA-induced transmodulation in Rat-1 cells. This increased rate of recovery is associated with a dephosphorylation of the EGF receptor and is antagonized by pretreatment of the cells with the serine/threonine phosphatase inhibitor okadaic acid. These results suggest that a cyclic AMP-mediated phosphatase can regulate binding capacity of the transmodulated EGF receptor.

METHODS

Cell Culture

Rat-1 embryo fibroblasts (obtained from Dr. Lynn Matrisian) were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% heat-inactivated bovine calf serum (Colorado Serum Company) in a humidified atmosphere (5% CO_2, 95% air) maintained at 37°C. Experimental cultures were grown to confluency in 35 mm Petri dishes using medium containing serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50 ug/ml streptomycin. Cells were used 3–4 days after density arrest.

EGF Binding

After PMA or PDGF treatment at 37°C, cells were rinsed three times on ice with cold binding buffer (Hanks' balanced salt solution supplemented with 0.1% bovine serum albumin and 25 mM HEPES, pH 7.4). Cells were then incubated for 3 hours at 4°C in 1.0 ml of binding buffer containing 2 ng of ^{125}I-EGF. After incubation, cells were washed three times with ice cold binding buffer. Cells were solubilized in two combined volumes of 0.5 ml of 0.1 N NaOH and 1% sodium dodecyl sulfate. Radioactive counts were measured using a gamma counter. Nonspecific binding was determined by the addition of 1 μg/ml unlabeled EGF to binding reaction. All binding data shown represent the specific binding average of at least duplicate samples.

EGF Receptor Immunoprecipitations

Cells were labeled for either 2 hours in 0.5 ml of phosphate free medium containing 0.25% platelet poor plasma (PPP) and 100 μCi of [^{32}P] orthophosphate or for 16–18 hours in 0.5 ml of methionine free medium supplemented with 0.25% PPP and 80 μCi of [^{35}S] methionine at 37°C. After treatment with 200 nM PMA or 50 ng/ml PDGF, cells were rinsed three times on ice with cold phosphate buffered saline solution. Cells were then lysed for 30 minutes at 4°C with 300 μl of lysis buffer containing 1.0% Triton, 20 mM HEPES, 10% glycerol, 50 mM NaF, and 1.0 mM vanadate, pH 7.2. Labeled EGF receptors were precipitated from cell lysates using a polyclonal antibody provided by Dr. Graham Carpenter. EGF receptors were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (7.5%). The gel was dried and labeled receptors

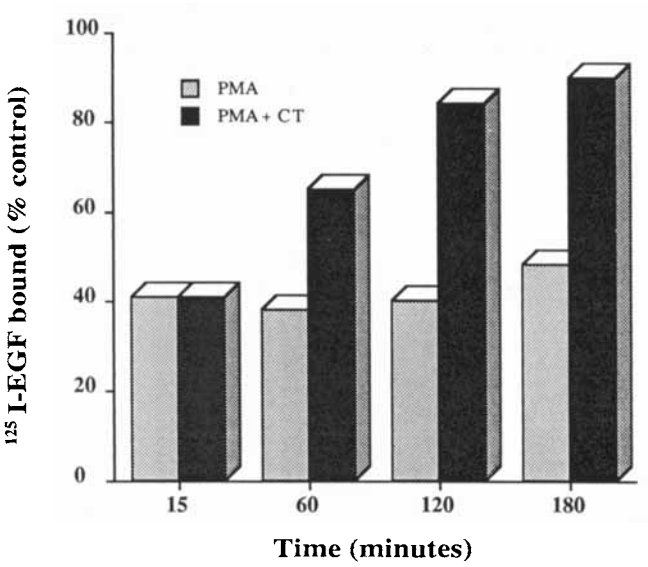

Fig. 1. Cholera toxin enhances recovery of EGF binding after PMA-induced transmodulation. Confluent Rat-1 fibroblasts were treated with 200 nM PMA or PMA and 1 μg/ml cholera toxin (PMA + CT) at 37°C for 15, 60, 120, and 180 minutes. Cells were then washed with cold binding buffer and incubated with 2 ng/ml of ^{125}I-EGF for three hours at 4°C. Radiolabeled EGF binding was measured in duplicate samples and nonspecific binding, determined by including 1 μg/ml of unlabeled EGF in binding medium, was subtracted from all values.

were visualized by autoradiography. Receptor phosphorylation was quantitated using densitometry scanning.

MATERIALS

PDGF (recombinant BB) was purchased from Amgen Biologicals (Thousand Oaks, CA). PMA was purchased from Sigma. PPP was prepared as previously described (Pledger et al., 1977). EGF was purified from mouse submaxillary glands as previously described (Savage and Cohen, 1972) and labeled with [^{125}I] as described (Markwell, 1982), using IODO-BEADS purchased from Pierce Chemical Co. EGF receptors were labeled with [^{32}P] orthophosphate purchased from New England Nuclear (Boston, MA) or [^{35}S] methionine purchased from ICN Biochemicals (Costa Mesa, CA).

RESULTS

Elevation of intracellular cyclic AMP concentration has been shown to have a variety of effects on phorbol ester-induced transmodulation in distinct cell lines, suggesting that cyclic AMP may influence the transmodulation pathway through a cell type specific mechanism. We therefore used the adenylate cyclase activator cholera toxin to determine the effect of cyclic AMP on PMA-stimulated transmodulation in Rat-1 fibroblasts. Density arrested Rat-1 cells were treated with 200 nM PMA for time periods up to 3 hours at 37°C. As shown in Figure 1, cells exposed to PMA for 15 minutes exhibited a rapid decrease in specific ^{125}I-EGF binding to approximately 40% of control values. Inhibition of binding was maximal within 60 minutes of PMA treatment, and receptor binding capacity did not increase significantly over a 3-hour time course. No significant decrease in binding was observed in quiescent cells refed with medium and 0.25% platelet poor plasma (data not shown).

When cells were treated with PMA in the presence of 1 μg/ml cholera toxin, the initial reduction of binding was not affected, as compared with those cells stimulated with PMA alone (Fig. 1). However, in PMA-treated cultures simultaneously exposed to cholera toxin, recovery of high affinity EGF binding was markedly enhanced by 1 hour. While cells receiving PMA alone exhibited only a slight increase in binding after 3 hours, cells incubated with PMA and cholera toxin recovered to 90% of control binding after the same time period. Cholera toxin alone had no significant effect on EGF binding (data not shown). These data are in contrast to previously reported results with Balb/c-3T3 cells, where cholera toxin delayed recovery of binding rather than enhance the recovery of receptor affinity after phorbol ester-induced transmodulation (Olashaw et al., 1986).

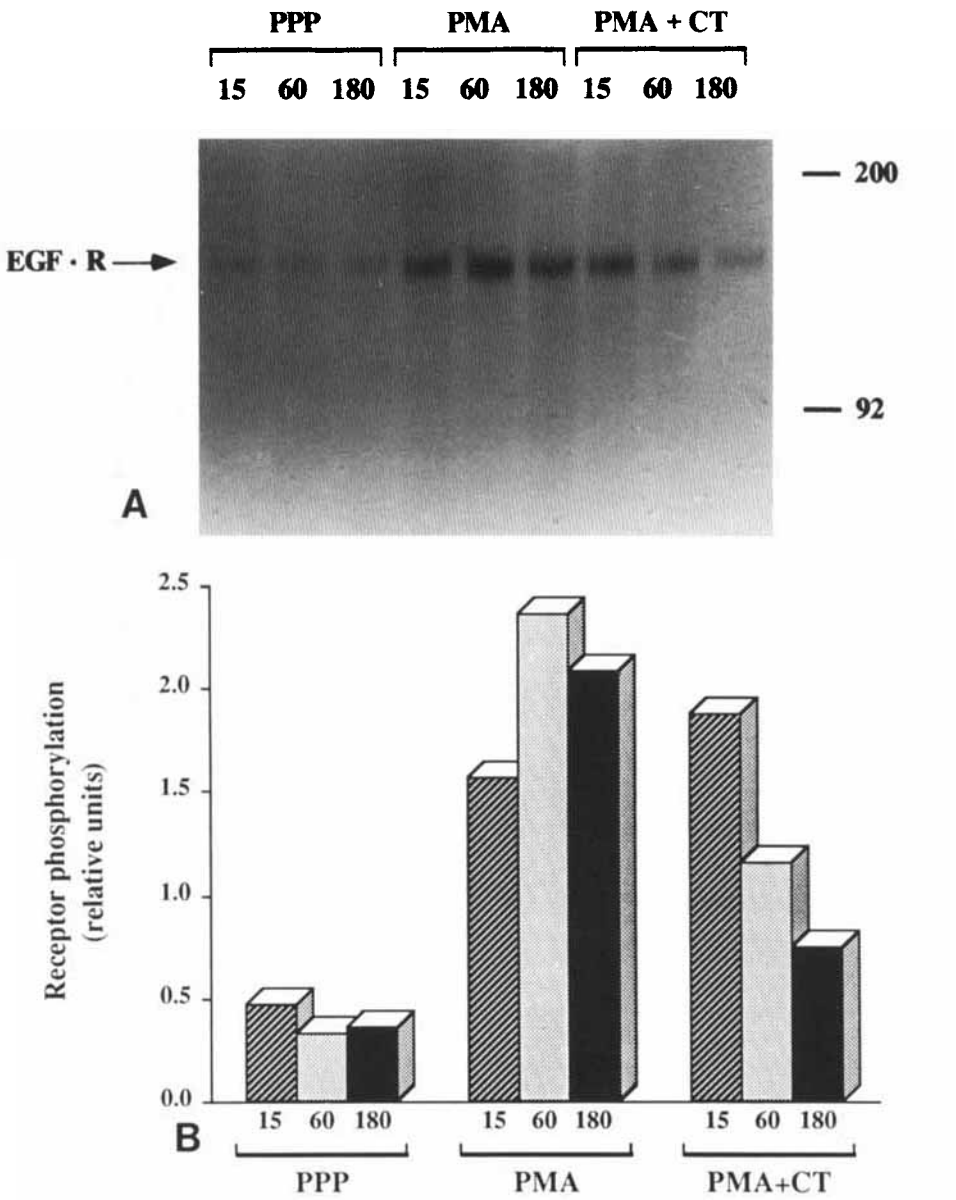

Fig. 2. Cholera toxin stimulates a decrease in EGF receptor phosphorylation after PMA-induced transmodulation. **(A)** Density arrested Rat-1 cells were labeled with 200 μCi/ml [^{32}P] orthophosphate in phosphate free medium supplemented with 0.25% PPP. PMA or PMA and cholera toxin (PMA + CT) were added directly to the medium after a 2 hour labeling period. Control cells (PPP) received no stimulation. Cells were lysed in triton-glycerol buffer after 15-, 60-, or 180-minute treatments, and an equal number of acid precipitable counts were immunoprecipitated with a polyclonal anti-EGF receptor antibody. Immunoprecipitated receptors were resolved on an SDS-polyacrylamide gel and visualized by autoradiography. **(B)** Receptor phosphorylation was quantitated using densitometry scanning.

If the observed decrease in EGF binding was a result of PMA-induced receptor phosphorylation, it might be expected that cholera toxin enhances the recovery of binding by further altering the phosphorylation state of the EGF receptor in PMA-transmodulated cells. To determine the effect of PMA and cholera toxin on the receptor phosphorylation state, quiescent Rat-1 cells were labeled in phosphate free medium with 200 μCi/ml of [^{32}P] orthophosphate for 2 hours. Cells were stimulated with PMA in the presence or absence of cholera toxin at concentrations used to characterize EGF binding inhibition. After the cells were solubilized, the EGF receptor was immunoprecipitated with a polyclonal antibody, electrophoresed on an SDS denaturing gel, and the gel was dried and autoradiographed. As shown in Figure 2, PMA induced

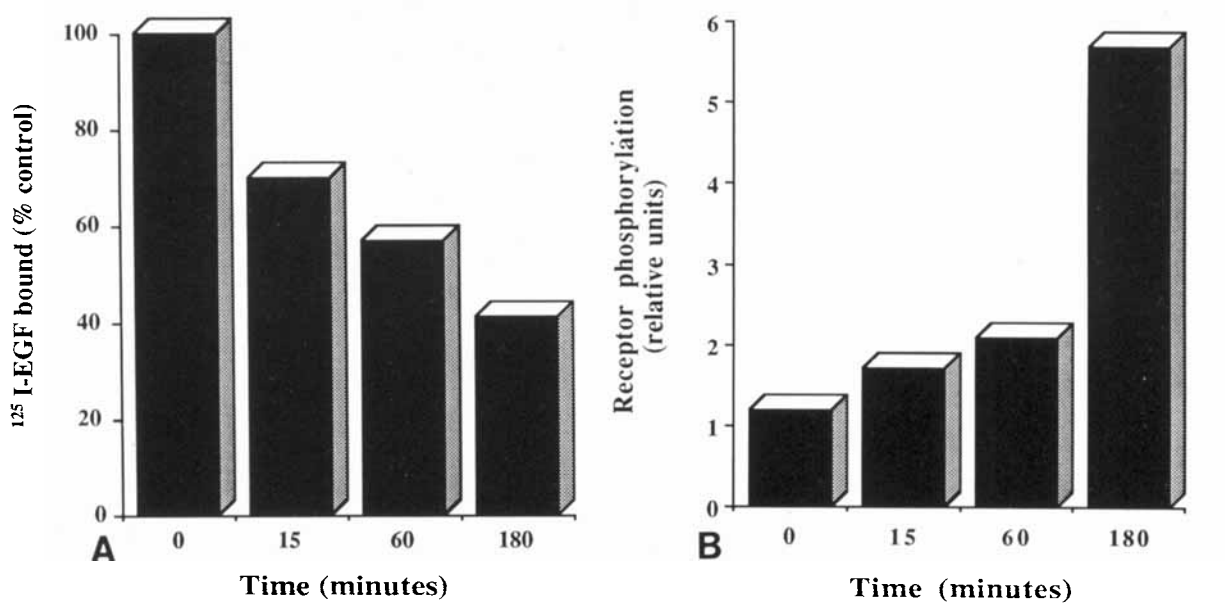

Fig. 3. Effect of PMA treatment at 4°C on EGF binding and receptor phosphorylation. **(A)** Quiescent Rat-1 cells were incubated at 4°C for 30 minutes in DME supplemented with 10% calf serum. Cells were then stimulated at 4°C by the addition of fresh, cold DME containing 0.25% PPP and 200 nM PMA. At 15, 60, and 180 minutes after stimulation, cells were washed and incubated for 3 hours at 4°C with ^{125}I-EGF. Specific binding is represented as the mean of duplicate samples. **(B)** Rat-1 cells were labeled with 200 μCi/ml [^{32}P] orthophosphate for 2 hours at 37°C. Cells were then placed on ice at 4°C and incubated for 30 minutes. PMA was added directly to medium and cells were lysed 15, 60, and 180 minutes after stimulation. An equal number of acid precipitable counts were immunoprecipitated with an anti-EGF receptor antibody, and proteins were resolved on an SDS-polyacrylamide gel. Receptors were visualized by autoradiography and phosphorylation was quantitated using laser densitometry.

a rapid phosphorylation of the EGF receptor which occurred within 15 minutes of treatment. This phosphorylation was sustained for at least 3 hours at a level comparable to that observed at 15 minutes. As determined by densitometry scanning, PMA stimulated a maximal 7.2-fold increase in receptor phosphorylation after 60 minutes, as compared with cells refed with control medium.

Just as cholera toxin did not affect the PMA-stimulated inhibition of EGF binding at 15 minutes, phosphorylation of the PMA-transmodulated EGF receptor was not decreased by the presence of cholera toxin at this time. However, by 60 minutes cells treated with PMA in the presence of cholera toxin showed a decrease in the amount of EGF receptor phosphorylation. Whereas labeling of the EGF receptor remained at maximal levels between 15 and 60 minutes in cells treated with PMA alone, receptor phosphorylation decreased approximately 38% during the same time period in cells receiving PMA and cholera toxin. Receptor phosphorylation further decreased between 1 and 3 hours in PMA/cholera toxin stimulated cells, although never reaching basal levels observed in quiescent cells. Thus, the time course of PMA-induced receptor phosphorylation closely paralleled that of PMA-stimulated inhibition of binding both in the presence and absence of cholera toxin. Cholera toxin by itself did not induce a change in the receptor phosphorylation state (data not shown).

It is interesting to note that inhibition of EGF binding elicited by PMA treatment at 4°C correlated with the degree of receptor phosphorylation much as it did at 37°C (Fig. 3). PMA treatment of cells at 4°C induced a steady decrease in binding which reached 40% of control values after 3 hours. Immunoprecipitation of [^{32}P] labeled EGF receptor showed a maximal 2.9-fold increase in receptor phosphorylation after the same 3-hour period which correlated directly with the time course of binding inhibition. These results further suggest a link between PMA-induced phosphorylation events and down regulation of EGF binding in Rat-1 fibroblasts.

Receptor immunoprecipitation of cells labeled overnight with 80 μCi/ml of [^{35}S] methionine revealed no obvious change in the total amount of EGF receptor protein after 1 hour of treatment with either PMA or PMA and cholera toxin (Fig. 4). In contrast, cells stimulated for 1

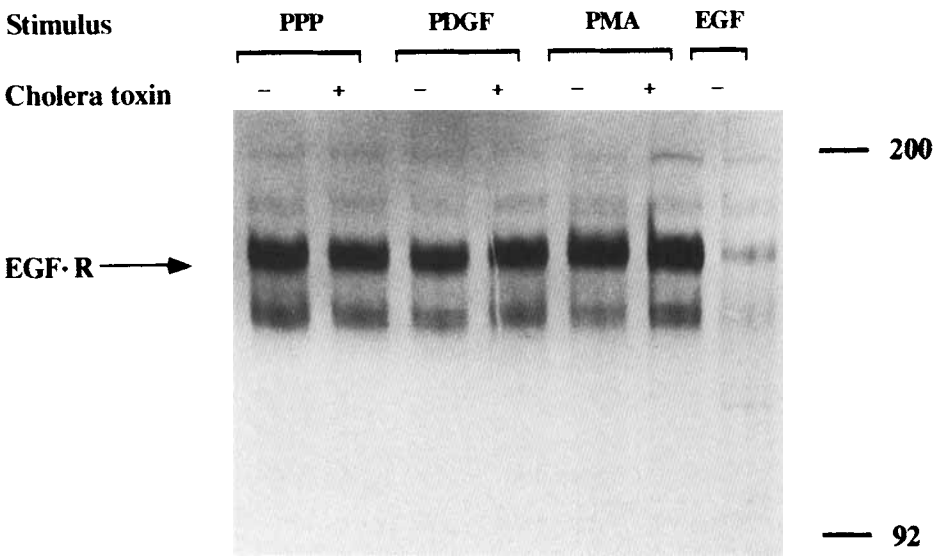

Fig. 4. One-hour PMA and PMA/cholera toxin treatments do not induce a change in total amount of EGF receptor protein. Rat-1 cells were labeled with 80 μCi/ml of [^{35}S] methionine for 18 hours in methionine free medium supplemented with 0.25% PPP. Cells were refed with DME containing unlabeled methionine and stimulated with PMA or PDGF in the presence and absence of cholera toxin toxin. Cells from lane 7 were treated with 50 ng/ml EGF. Control cells (PPP) were refed with DME and 0.25% PPP alone or in the presence of cholera toxin. After a 1-hour treatment, cells were lysed and an equal number of acid precipitable counts were incubated with antibody. Immunoprecipitated receptors were resolved on an SDS-polyacrylamide gel and visualized using autoradiography.

hour with EGF exhibited almost complete down regulation of the labeled receptor. These results suggest that neither the PMA-stimulated decrease in binding nor the cholera toxin-induced decrease in receptor phosphorylation is attributable to a reduction of receptor number associated with degradation of EGF receptor protein. Rather, it appears that PMA- and cholera toxin-mediated changes in receptor properties can be accounted for by biochemical modifications of the EGF receptor or an associated regulatory protein. Platelet-derived growth factor (PDGF), a well-characterized transmodulating agent which increases EGF sensitivity in a number of cell lines, also did not induce an apparent reduction in EGF receptor protein.

In order to investigate whether or not cholera toxin stimulated an increased rate of binding recovery through the activation of a protein phosphatase, we performed the following study. Okadaic acid, a non-phorbol tumor promoter which inhibits serine/threonine phosphatases PP1 and PP2A (Hescheler et al., 1988; Haystead et al., 1989), was used to determine the effect of phosphatase activity in the cholera toxin-induced recovery of EGF binding after transmodulation. Cells were pretreated for 2 hours with okadaic acid, and specific binding of radiolabeled EGF was measured after PMA or PMA/cholera toxin treatments. In those cells exposed to PMA after okadaic acid pretreatment, EGF binding decreased to approximately 30% of untreated control values at 15 minutes (Fig. 5). A slight but reproducible increase was observed at 60 minutes before binding again decreased to 15% at 3 hours. Thus, okadaic acid pretreatment potentiated the PMA-induced reduction of EGF binding. Treatment of cells with okadaic acid alone resulted in a small but reproducible increase in EGF binding at 1 hour before decreasing to 80–90% of control levels by 3 hours (data not shown).

Cells stimulated by PMA and cholera toxin after okadaic acid pretreatment showed an initial decrease in binding to approximately 45% of untreated control values. Binding increased slightly at 1 hour before again decreasing to 45% by 3 hours. In contrast, EGF binding recovered to 90% of control values in cells exposed to PMA and cholera toxin without okadaic acid pretreatment. Thus, the cholera toxin-stimulated increase in rate of binding recovery was antagonized in cells pretreated with okadaic acid. Moreover, we have observed that okadaic acid pretreatment blocked the cholera toxin-induced dephosphorylation of the EGF receptor after

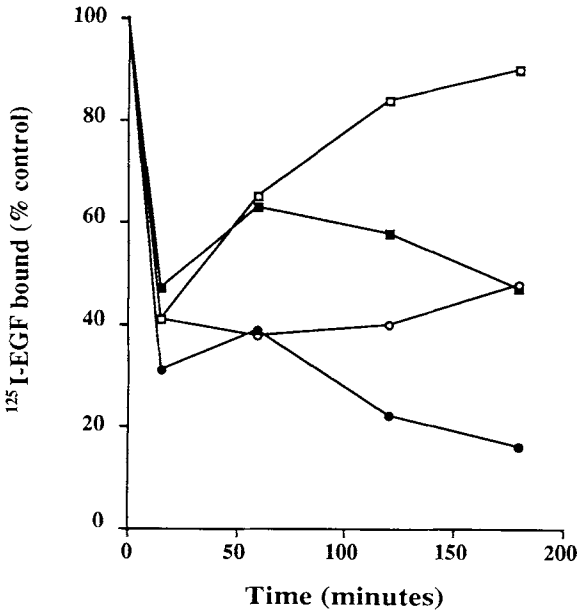

Fig. 5. Okadaic acid blocks the cholera toxin-induced recovery of binding after PMA-stimulated transmodulation. Quiescent Rat-1 fibroblasts were incubated for 2 hours at 37°C in medium containing 0.25% PPP and 240 ng/ml okadaic acid. Pretreated cells were stimulated by the addition of PMA (●) or PMA and cholera toxin (■) directly to the medium. Nontreated control cells were refed with DME supplemented with 0.25% PPP and PMA (○) or PMA and cholera toxin (□). At 15, 60, 120, and 180 minutes after stimulation, cells were washed and incubated with ^{125}I-EGF at 4°C. The specific binding reported represents the average of duplicate samples.

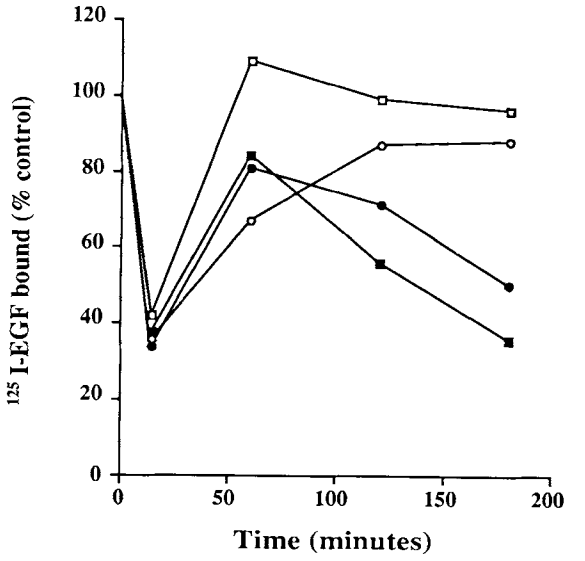

Fig. 6. Effects of cholera toxin and okadaic acid on EGF binding after PDGF-induced transmodulation. Density arrested Rat-1 cells were pre-exposed to okadaic acid for 2 hours at 37°C. Pretreated cells were stimulated by the addition of 50 ng/ml PDGF (●) or PDGF and cholera toxin (■). Nontreated cells were stimulated with PDGF (○) or PDGF and cholera toxin (□) in the absence of okadaic acid. ^{125}I-EGF binding was determined after treatment at the times indicated.

PMA-stimulated transmodulation, further suggesting that cholera toxin induces a phosphatase activity which acts upon the transmodulated EGF receptor (data not shown).

Platelet derived growth factor (PDGF) has previously been shown to decrease radiolabeled EGF binding in many cell lines and to stimulate increased phosphorylation of the EGF receptor (Wrann et al., 1980; Davis and Czech, 1985a). However, PDGF can transmodulate the EGF receptor through a protein kinase C independent pathway distinct from phorbol ester (Olashaw et al., 1986). We therefore determined whether or not cholera toxin could enhance recovery of EGF binding in cells transmodulated by PDGF. As shown in Figure 6, Rat-1 cells exposed to 50 ng/ml PDGF exhibited a decrease in specific EGF binding to approximately 35% of control values at 15 minutes. Although receptor binding capacity recovered to 88% of control values at 3 hours in cells receiving PDGF alone, binding of the transmodulated receptor fully recovered to 100% within 60 minutes in cells receiving PDGF and cholera toxin. Thus, cholera toxin elicited a qualitatively similar binding response in cells transmodulated by platelet-derived growth factor, as compared with results observed in cells treated with PMA. Furthermore, the cholera toxin-stimulated increase in the rate of binding recovery correlated with a decrease in the amount of receptor phosphorylation in cells transmodulated by PDGF (Fig. 7). Whereas PDGF alone induced a maximal 3.0-fold increase in phosphorylation of the EGF receptor at 1 hour, cells treated with PDGF in the presence of cholera toxin showed virtually no increase in receptor phosphorylation over control cells after the same time period. Hence, PDGF-treated cells, which recovered full binding capacity more rapidly in the presence of cholera toxin than PMA-treated cells, also returned to basal levels of phosphorylation in response to cholera toxin more quickly than PMA-stimulated cultures.

We then determined whether the inhibition of a cyclic AMP-mediated phosphatase in Rat-1 cells could alter normal EGF binding during PDGF-induced transmodulation. Although a significant transient increase in binding was observed between 15 minutes and 1 hour, okadaic acid effectively blocked complete recovery of EGF binding in both PDGF and PDGF/cholera toxin-

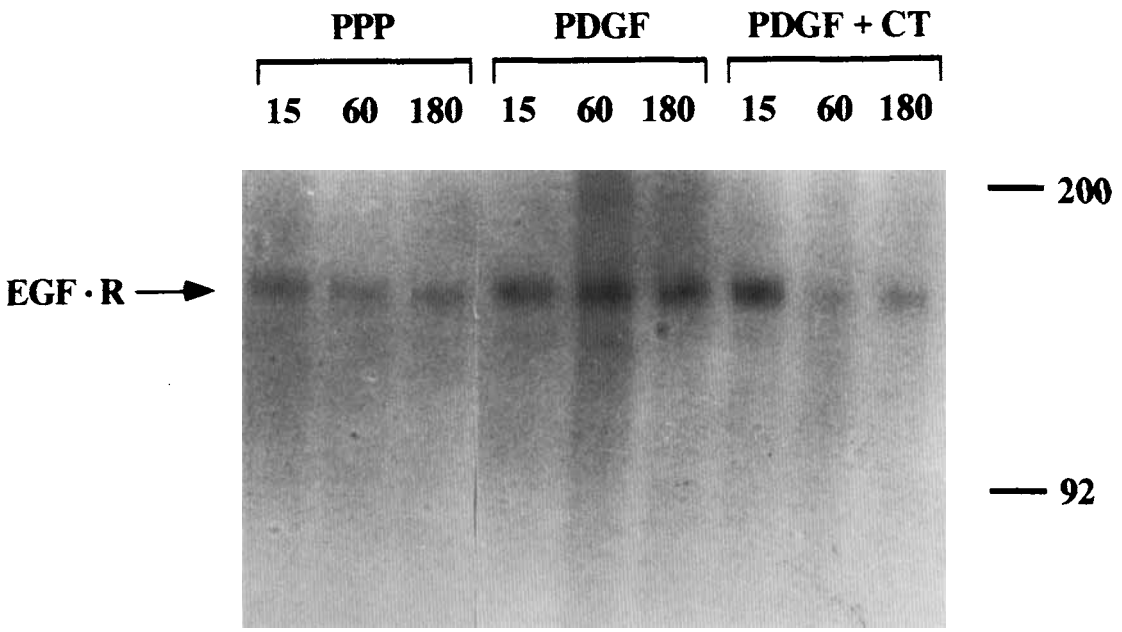

Fig. 7. Cholera toxin stimulates a decrease in EGF receptor phosphorylation after PDGF-induced transmodulation. Rat-1 cells were labeled with [^{32}P] orthophosphate for 2 hours in phosphate free medium supplemented with 0.25% PPP. PDGF or PDGF and cholera toxin (PDGF + CT) were added directly to the medium and cells were harvested 15, 60, and 180 minutes after stimulation. Control cells (PPP) received no stimulation. The EGF receptor was immunoprecipitated and visualized as previously described.

treated cells after 3 hours (Fig. 6). These data indicate that dephosphorylation is an important regulatory mechanism involved in the normal recovery of binding after PDGF transmodulation, as well as in the enhanced return of binding stimulated by cholera toxin treatment. However, we cannot determine from these results whether or not this event involves dephosphorylation of the EGF receptor or an associated regulatory protein. The reason for the increase in binding seen at 60 minutes in cells pretreated with okadaic acid and stimulated with PDGF or PDGF/cholera toxin is unclear, although it may reflect a need for synthesis of such an intermediate required in the PDGF transmodulation pathway (see Discussion).

DISCUSSION

The ability of phorbol esters to inhibit high affinity EGF binding in a variety of cell types is well documented (Lee and Weinstein, 1978; Shoyab et al., 1979). However, the mechanism by which transmodulation of the EGF receptor occurs is unclear. It has previously been demonstrated that a mutation in the cytoplasmic domain of the receptor can cause the loss of high affinity binding (Prywes et al., 1986), suggesting that a modification of the receptor in this region may be responsible for the phorbol ester-induced reduction of EGF binding observed in vivo. One such potential modification is phosphorylation. Treatment of intact cells with phorbol esters such as PMA increases phosphorylation of the EGF receptor above basal levels at several serine and threonine residues, as well as unique phosphorylation at threonine 654 (Davis and Czech, 1985). Other agents which inhibit EGF binding, such as calcium ionophore A23187, exogenous ATP, non-phorbol tumor promoter thapsigargon, and PDGF, also induce EGF receptor phosphorylation (Friedman et al., 1989; Hosoi and Edidin, 1989; Davis and Czech, 1985). In Rat-1 cells, inhibition of serine/threonine dephosphorylation events with okadaic acid potentiates PMA-induced transmodulation and blocks the cholera toxin-enhanced recovery of EGF binding. These data suggest that PMA induction of phosphorylation events is an important regulatory mechanism in the control of receptor affinity and specifically suggest that a corresponding dephosphorylation event is necessary for complete recovery of binding after receptor transmodulation. This model is consistent with the finding that staurosporine, a general kinase

inhibitor, antagonizes the decrease in EGF binding induced by several transmodulating agents, including phorbol ester (Friedman et al., 1990).

EGF receptors phosphorylated by protein kinase C, the cellular receptor for phorbol esters, have previously been shown to act as substrates for a membrane associated serine/threonine phosphatase (Cochet et al., 1984). However, the relevance of this phosphatase to the transmodulation mechanism is not known. Protein kinase C and an alkaline phosphatase have been shown to act antagonistically in the regulation of the insulin receptor tyrosine kinase activity in vitro, although no inhibition of binding was observed (Takayama et al., 1988). In our experiments with Rat-1 fibroblasts, the adenylate cyclase activator cholera toxin stimulated a decrease in the phosphorylation of EGF receptors transmodulated by PMA or PDGF. Cholera toxin could alter the phosphorylation state of the receptor by inactivating a protein kinase, thereby preventing rephosphorylation of the receptor, or by activating a phosphatase and thus stimulating direct removal of phosphate from the protein. Although a known kinase activator, cyclic AMP has been shown previously to upregulate phosphatase activity 2,000-fold in mouse L cells (Firestone and Heath, 1981).

The non-phorbol tumor promoter, okadaic acid, is a toxic derivative of a 38 carbon fatty acid which inhibits serine/threonine phosphatases 1 and 2A through the specific binding and ensuing inactivation of these enzymes (Tachibana et al., 1981; Suganuma et al., 1989). A 2-hour pretreatment of Rat-1 cells with okadaic acid antagonized the cholera toxin-stimulated recovery of EGF binding, indicating that cholera toxin treatment and the subsequent elevation of cyclic AMP levels are acting to mediate a phosphatase activity which can regulate binding of the transmodulated receptor in Rat-1 cells. In addition, we have observed that exposure of cells to okadaic acid blocked the cholera toxin-induced dephosphorylation of the transmodulated receptor, suggesting that the EGF receptor itself is a substrate for this cyclic AMP-mediated phosphatase.

It has previously been demonstrated that phorbol esters and PDGF act through distinct pathways in the transmodulation of the EGF receptor. Down regulation of protein kinase C by chronic exposure to PMA does not affect the PDGF stimulated decrease in EGF binding (Olashaw et al., 1986). Similarly, treatment of cells with chlorpromazine or neomycin, inhibitors of C-kinase activation, fails to block PDGF induced transmodulation (Walker and Burgess, 1988). Yet the PDGF receptor tyrosine kinase does not phosphorylate the EGF receptor directly (Davis and Czech, 1985a). Therefore, it is likely that an intermediate distinct from protein kinase C is required for the PDGF regulation of EGF receptor affinity.

Although different kinases can be involved in the separate transmodulation pathways, cholera toxin induced a dephosphorylation of the EGF receptor in both PMA and PDGF stimulated Rat-1 cells. Furthermore, cholera toxin activation of a Rat-1 phosphatase was associated with an increased rate of binding recovery in cells transmodulated with either PMA or PDGF. These results suggest that unique pathways may share a common mechanism of receptor regulation through the induction of specific phosphorylation/dephosphorylation events. The observation that the phosphatase inhibitor okadaic acid antagonized complete recovery of EGF binding in cells treated with PMA or PDGF in the presence and absence of cholera toxin further supports this hypothesis.

Under all treatment conditions studied, EGF binding increased to varying extents between 15 and 60 minutes after okadaic acid pretreatment before again decreasing to a level comparable to the initial binding inhibition observed at 15 minutes. Thus, transmodulated Rat-1 cells exhibited a biphasic response to okadaic acid in which recovery of binding during the first hour of treatment was independent of serine/threonine dephosphorylation events. The reason for this transient increase is not known. However, a similar pattern of binding recovery has previously been reported in Balb/c-3T3 fibroblasts after PDGF-induced transmodulation and in Balb/c-3T3 cells transformed by $pp60^{v\text{-}src}$ (Olashaw *et al.*, 1986; Gray and Macara, 1988). In these systems, continued down regulation of EGF binding after the initial rapid decrease was inhibited by cyclohexamide, suggesting that maintenance of the transmodulated state was dependent upon protein synthesis. The synthesis of similar regulatory proteins in Rat-1 cells may be required to effect the observed secondary decrease in receptor binding capacity.

Recent studies mutating the EGF receptor at the major sites of PMA- and PDGF-induced

phosphorylation have revealed that transmodulation occurs independently of these sites (Countaway et al., 1990). Two models are consistent with the findings of this study and the data reported here. First, a minor site of phosphorylation on the EGF receptor could regulate receptor binding. Cell surface EGF receptors exist in two separate affinity states (Magun et al., 1980; King and Cuatrecasas, 1982). High affinity receptors typically comprise 1–10% of the total receptor population. Thus, a phosphorylation specific for high affinity receptors would likely occur with a low stoichiometry. Site specific phosphorylation of this type could be masked in immunoprecipitations by the much greater degree of phosphorylation at major non-response sites, such as threonine 654 and threonine 669. After PDGF treatment of Rat-1 cells, EGF binding recovered without an obvious dephosphorylation of the receptor. However, immunoprecipitation assays may not be sensitive enough to detect the dephosphorylation of specific minor sites which regulate binding of a subpopulation of receptors. In support of this model, Countaway et al. (1990) report in their mutagenesis study that [^{32}P] phosphate labeling of all identified minor sites of phosphorylation on the mutant receptor increased after phorbol ester treatment.

Secondly, phosphorylation of a receptor associated regulatory protein rather than the EGF receptor itself may control receptor affinity. It is possible that phosphorylation and subsequent dephosphorylation regulate a protein which interacts with the EGF receptor. Such a protein must associate with the receptor in a stable fashion, as membranes purified from transmodulated cell lysates remain inhibited in their binding of EGF (Fearn and King, 1985). The finding that activated protein kinase C phosphorylates and transmodulates binding of solubilized, purified EGF receptors suggests that no intermediate is necessary for the phorbol ester-induced pathway. In our experiments, kinetics of receptor phosphorylation closely paralelled that of binding inhibition in PMA-treated cells. However, PDGF stimulation of intact cells does not induce tyrosine phosphorylation of the EGF receptor, suggesting that an intermediate kinase is necessary for the PDGF induction of EGF receptor phosphorylation. Phosphorylation and subsequent dephosphorylation of such an intermediate may be important in the regulation of its catalytic activity or its association with the EGF receptor.

In Rat-1 cells treated with PDGF, alterations in the EGF receptor phosphorylation state did not strictly correlate with changes in EGF binding. Yet, pretreatment of PDGF-transmodulated cells with the phosphatase inhibitor, okadaic acid, antagonized complete recovery of binding. Taken together, these results suggest that, at least in the PDGF-induced transmodulation pathway, phosphorylation/dephosphorylation events may regulate a protein other than the EGF receptor, which, in turn, governs receptor binding capacity. Further studies will be required to elucidate the mechanism of EGF receptor transmodulation and distinguish between these two hypotheses.

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