Platelet-derived growth factor mimics phorbol diester action on epidermal growth factor receptor phosphorylation at threonine-654

(protein kinase C/tyrosine kinase/diacylglycerol/cell proliferation)

ROGER J. DAVIS AND MICHAEL P. CZECH

Department of Biochemistry, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605

ABSTRACT   Addition of platelet-derived growth factor (PDGF) to quiescent WI-38 human fetal lung fibroblasts mimics the effect of tumor-promoting phorbol diesters to inhibit the high-affinity binding of ^{125}I-labeled epidermal growth factor (^{125}I-EGF). PDGF, like phorbol diesters, was found to increase the phosphorylation state of EGF receptors immunoprecipitated from intact fibroblasts that were labeled to equilibrium with [^{32}P]phosphate. Phosphoamino acid analysis of the EGF receptors indicated that both PDGF and phorbol diesters increased the level of [^{32}P]phosphoserine and [^{32}P]phosphothreonine. Phosphopeptide mapping of the EGF receptor demonstrated that PDGF increased the phosphorylation of several sites and induced the phosphorylation of a site that was not observed to be phosphorylated on EGF receptors isolated from control cells. This latter phosphorylation site on the EGF receptor was identified as threonine-654, previously shown to be phosphorylated in response to phorbol diesters in intact cells or by purified protein kinase C in vitro. Further, it was observed that PDGF mimicked the action of phorbol diesters to inhibit the EGF-dependent tyrosine phosphorylation of the EGF receptor in [^{32}P]phosphate-labeled fibroblasts. These results are consistent with the hypothesis that increases in diacylglycerol and Ca^{2+} levels caused by addition of PDGF to fibroblasts activate protein kinase C and that this kinase, at least in part, mediates the effect of PDGF on the phosphorylation of the EGF receptor. The data further suggest that protein kinase C may play an important role in the regulation of cellular metabolism and proliferation by PDGF.

Platelet-derived growth factor (PDGF) is a potent polypeptide mitogen that is released into the blood during platelet lysis after tissue injury. The full mitogenic effect of PDGF is only expressed in the presence of other growth factors such as epidermal growth factor (EGF) and insulin (1). Interestingly, this effect is also characteristic of tumor-promoting phorbol diesters such as 4β-phorbol 12β-myristate 13α-acetate (PMA), which stimulate the growth of quiescent fibroblasts synergistically with EGF and insulin (2, 3). Another striking similarity between the actions of PDGF and phorbol diesters is a rapid inhibition of ^{125}I-labeled EGF (^{125}I-EGF) binding to the receptors of cultured cells (4–12). In view of the similarity of these actions of PDGF and PMA on the EGF receptor, we considered the possibility that these two agents may have a similar mechanism of action.

It has been reported that PMA causes the phosphorylation of the EGF receptor on serine and threonine residues, and it has been suggested that this phosphorylation is causally related to the effect of PMA on the EGF receptor (13–15). Phosphopeptide mapping of the EGF receptor has revealed that the phosphorylation state of several sites is increased after treatment of cells with phorbol diester (13, 15, 16). One

of these sites was observed to be uniquely phosphorylated on the EGF receptor isolated from PMA-treated cells (13, 15, 16). This site was also found to be phosphorylated by the putative phorbol diester receptor protein kinase C (kinase C) (17–20) in vitro (13). Sequence analysis of the tryptic phosphopeptides that contain this site demonstrated that it is threonine-654 (16, 21) that is located close to the predicted transmembrane region of the EGF receptor on the cytoplasmic side of the plasma membrane (22). This site is in a position between the EGF-binding and protein kinase domains of the receptor and, therefore, could have an important role in regulating receptor functions such as internalization, binding affinity, and tyrosine kinase activity. It has been shown that phosphorylation of the EGF receptor in vitro on threonine-654 can account for the ability of phorbol diesters to inhibit the tyrosine kinase activity of the EGF receptor (13).

There is reason to suspect that the bioactions of both phorbol diesters and PDGF may involve kinase C activation, at least in part. A rapid effect of PDGF on fibroblasts is to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate which results in an increase in the level of diacylglycerol and inositol 1,4,5-trisphosphate (23, 24). The inositol 1,4,5-trisphosphate causes the release of Ca^{2+} from intracellular stores and results in an increased free Ca^{2+} concentration in the cytoplasm (24, 25). The dual action of PDGF to increase the level of diacylglycerol and free Ca^{2+} would be expected to stimulate the activity of kinase C. The effect of PMA to stimulate the activity of kinase C is similar to that caused by diacylglycerol (20), and it has been shown that PMA and diacylglycerol interact at the same binding site on kinase C (26). Furthermore, addition of exogenous diacylglycerol to cultured cells causes changes in the apparent affinity and phosphorylation state of the EGF receptor that are the same as those observed when the cells are treated with PMA (27, 28). These observations raise the possibility that the EGF receptor may be physiologically regulated by changes in the activity of kinase C caused by diacylglycerol.

The aim of the experiments presented in this report was to test the hypothesis that stimulation of the activity of kinase C by PDGF and PMA may be a common site of action of these agents. Our approach was to examine the ability of PDGF in intact cells to cause phosphorylation of the EGF receptor at threonine-654, the known kinase C phosphorylation site. We present results here that demonstrate such an effect, which provides direct support for the hypothesis tested.

EXPERIMENTAL PROCEDURES

Materials. EGF was prepared as described (29, 30) and iodinated by using the immobilized lactoperoxidase meth-

Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PMA, 4B-phorbol 12β-myristate 13α-acetate; TPCK-treated trypsin, tosylphenylalanyl chloromethyl ketone-treated trypsin; kinase C, protein kinase C.

od (31). Tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington. [^{32}P]Phosphate and Na^{125}I were from New England Nuclear and Amersham, respectively. Protein A-Sepharose CL-4B was obtained from Pharmacia. PDGF purified to homogeneity (32) was a gift from C. H. Heldin.

Methods. *Cell culture.* WI-38 fibroblasts (American Type Culture Collection CCL75) were maintained in minimal essential medium supplemented with 10% fetal bovine serum. The cells were seeded and grown to confluence in 2 days. The medium was replaced 1 day after confluence, and the cells were then allowed to grow for 3 days before use. In some experiments, the cells were labeled with [^{32}P]phosphate for 24 hr by incubation with phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 3 mCi (1 Ci = 37 GBq) of [^{32}P]phosphate per ml.

Measurement of the binding of ^{125}I-EGF to cell monolayers. WI-38 cells in 35-mm wells were incubated in a medium containing 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl$_2$, 1 mM MgSO$_4$, 10 mM glucose, 25 mM Hepes (pH 7.4), and 1% (wt/vol) bovine serum albumin. The cells were treated with PDGF or PMA for 30 min at 37°C and then incubated at 0°C for 4 hr with 100 pM ^{125}I-EGF. The monolayers were then carefully washed four times with cold medium and solubilized with 0.4 M NaOH. Radioactivity was quantitated with a Beckman γ counter. Nonspecific binding was estimated in incubations containing 100 nM EGF.

Immunoprecipitation of EGF receptors. The cell monolayers were washed once, and the cells were lysed with 1.5% Triton X-100/1% Na deoxycholate/0.1% NaDodSO$_4$/0.5 M NaCl/5 mM EDTA/50 mM NaF/100 μM Na$_3$VO$_5$/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/25 mM Hepes, pH 7.8. The volume of lysis buffer used was 1.5 ml and 4 ml in experiments with 35-mm and 100-mm dishes, respectively. The lysate was clarified by centrifugation at 4°C for 30 min at 100,000 × *g*. The supernatant was then mixed with anti-EGF receptor antiserum (1:1000 dilution) and incubated for 60 min at 22°C. The immune complexes were then collected by adding protein A-Sepharose CL-4B (20 μl packed beads per ml). After a further 60 min, the protein A-Sepharose was extensively washed with lysis buffer and finally washed with 0.1% NaDodSO$_4$/0.2% Triton X-100/25 mM Hepes, pH 7.8.

Phosphopeptide mapping. Immunoprecipitated EGF receptors in a vol of 100 μl were reduced by the addition of 80 μl of 10% NaDodSO$_4$/14 mM dithiothreitol and heated to 60°C for 15 min. The receptors were then alkylated with 40 μl of 0.4 M iodoacetamide dissolved in 0.25 M Tris·HCl (pH 8.6). After 15 min of incubation at room temperature, 100 μl of 25% 2-mercaptoethanol/75% glycerol was added, and the samples were heated to 60°C for 15 min. The reduced and alkylated receptors were then resolved from other proteins on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO$_4$. The gel was fixed and dried, and the EGF receptors were located by autoradiography. The gel slices containing the EGF receptor were excised, swollen in water, and transferred to 100 μl of 100 mM N-ethylmorpholine (pH 8.3) containing 3 μg of TPCK-treated trypsin. The gel slice was incubated at 37°C for 5 hr before a second addition of 3 μg of trypsin was made. After 24 hr the gel slice was extensively washed with buffer, and the combined supernatants were lyophilized. The phosphopeptides recovered were dissolved in water and applied to a 20 × 10 cm cellulose-coated thin-layer plate (Machery & Nagel). The phosphopeptides were resolved by electrophoresis in 1% ammonium carbonate (pH 8.9) for 2 hr at 400 V (anode at left) and ascending chromatography with pyridine/water/acetic acid/1-butanol, 50:60:15:75 (vol/vol), as solvent.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed by the method of Hunter and Sefton (33) as described (15).

RESULTS

In preliminary experiments the effect of PDGF on quiescent WI-38 human fetal lung fibroblasts was investigated. The fibroblasts were found to respond to PDGF in mitogenic assays in which the incorporation of radioactivity from [*methyl*-^{3}H]thymidine into acid-insoluble material was measured (not shown). PDGF also was found to inhibit the binding of ^{125}I-EGF to monolayers of these cells (Fig. 1) in a manner similar to that reported for other fibroblasts (4–9). Addition of the tumor promoter PMA to the fibroblasts similarly caused a decrease in the binding of ^{125}I-EGF (Fig. 1). The inhibition of ^{125}I-EGF binding in response to both PMA and PDGF was observed to be greater at low compared with high ^{125}I-EGF concentrations (not shown) as described in other cell lines (4–12).

To study the effect of PDGF on the phosphorylation state of the EGF receptor, we labeled postconfluent WI-38 fibroblasts with [^{32}P]phosphate by incubation in phosphate-free Dulbecco's modified Eagle's medium supplemented with 3 mCi of [^{32}P]phosphate per ml for 24 hr. The cells were then treated with and without PDGF or PMA for 40 min at 37°C. EGF was added to some incubations for the last 5 min. Subsequently, the cells were lysed, and the EGF receptors were extracted, immunoprecipitated, and electrophoresed on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO$_4$. An autoradiogram of the fixed, stained, and dried gel is presented in Fig. 2. EGF, as expected, increased the phosphorylation state of the EGF receptor. The increase was quantitated by densitometry of the autoradiograph and was calculated to be 2.1-fold. PMA caused a 3-fold increase in the phosphorylation state of the EGF receptor, whereas addition of PDGF to the WI-38 cells caused a 1.5-fold increase in the phosphorylation state of the EGF receptor (Fig. 2). Therefore, addition of PDGF to fibroblasts not only increases the phosphorylation state of the receptor for PDGF as reported (34, 35) but also causes an increase in the phosphorylation state of the EGF receptor.
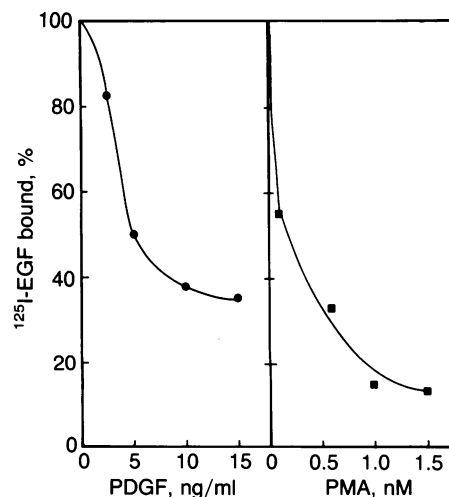

FIG. 1. The effect of PDGF and PMA on the binding of ^{125}I-EGF to WI-38 fibroblasts. Confluent WI-38 fibroblasts in 35-mm dishes were treated with different concentrations of PDGF (*Left*) or PMA (*Right*) for 30 min at 37°C. The binding of 100 pM ^{125}I-EGF to the monolayers at 0°C was then measured. Nonspecific binding was determined in parallel incubations with 100 nM EGF. The results are presented as the average of three experiments.
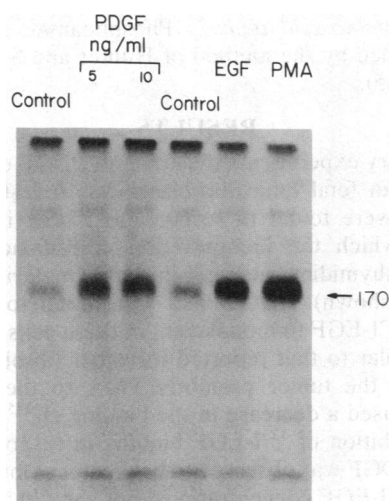
FIG. 2. Effect of PDGF, EGF, and PMA on the phosphorylation state of the EGF receptor in intact WI-38 fibroblasts. The cells were grown to confluence in 35-mm dishes. Three days after confluence, the cells were transferred to 1 ml of phosphate-free Dulbecco's modified Eagle's medium containing 3 mCi of [^{32}P]phosphate (New England Nuclear) and incubated for 24 hr. The cells were then treated for 30 min with and without PDGF or 10 nM PMA. After this incubation, 10 nM EGF was added to some wells for 5 min. The cells were then solubilized, and the EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis in the presence of 0.1% NaDodSO$_4$. The gel was fixed, stained, dried, and autoradiographed by using Kodak X-OMAT AR film and a Dupont Lightning Plus enhancing screen at −70°C. An autoradiograph that was exposed for 48 hr is shown.

The effect of PDGF on the phosphorylation state of the WI-38 EGF receptor was further characterized by phospho-amino acid analysis (Fig. 3). The EGF receptor isolated from control WI-38 cells contained [^{32}P]phosphoserine and [^{32}P]phosphothreonine. PDGF enhanced the level of these two phosphoamino acids. This action is similar to that observed with PMA (Fig. 3). In contrast, EGF caused the appearance of [^{32}P]phosphotyrosine in addition to increasing the level of [^{32}P]phosphoserine and [^{32}P]phosphothreonine (Fig. 3). As the purified PDGF preparations we used did not cause tyrosine phosphorylation of the EGF receptor, we conclude that the effect of PDGF cannot be accounted for by contamination with an EGF-like peptide present in platelet lysates (36). Furthermore, addition of the PDGF preparation
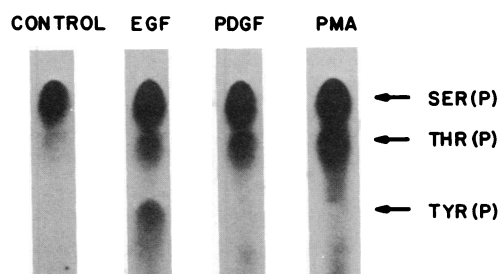

FIG. 3. Phosphoamino acid analysis of the EGF receptor isolated from intact WI-38 fibroblasts. Confluent WI-38 cells were labeled with [^{32}P]phosphate and treated with PMA, PDGF, or EGF as described in the legend to Fig. 2. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The EGF receptors were excised from the gel, extracted, precipitated with trichloroacetic acid, partially hydrolyzed in 6 M HCl at 110°C for 60 min, and electrophoresed at pH 3.5 on cellulose-coated thin-layer sheets. An autoradiograph (8-day exposure) of the dried sheet is shown. Phosphoamino acids were identified by staining standards mixed with each sample with ninhydrin.

to Swiss 3T3 membranes was observed to cause the phosphorylation of a M_r 185,000 band, which has been identified as the PDGF receptor (37–40), but did not affect the phosphorylation state of the M_r 170,000 EGF receptor (not shown). We conclude that PDGF causes the phosphorylation of the EGF receptor in WI-38 cells on serine and threonine residues in a manner that is similar to that observed when the cells are treated with tumor-promoting phorbol diesters such as PMA. However, the magnitude of the response of the cells to PDGF is not as great as that to PMA.

To investigate the sites phosphorylated on the EGF receptor after treatment of WI-38 cells with PMA or PDGF, the receptors were digested with trypsin and the phosphopeptides obtained were resolved by thin-layer electrophoresis and chromatography (Fig. 4). Phosphopeptide maps of the EGF receptor isolated from cells treated with PMA contained four additional peptides that were not observed in maps of the EGF receptor isolated from control cells. Phosphopeptides 1, 2, and 3 have been identified as Thr(P)-Leu-Arg, Arg-Thr(P)-Leu-Arg, and Lys-Arg-Thr(P)-Leu-Arg, respectively (16, 21). The fourth peptide was identified as Lys-Arg-Thr(P)-Leu-Arg-Arg by showing that phosphopeptide 4 comigrated during the peptide mapping procedure with a synthetic peptide of this structure (16). Therefore, the phosphopeptides 1, 2, 3, and 4 are structurally related and are derived from the EGF receptor by incomplete proteolytic digestion. This phosphorylation site has been identified as threonine-654 (16, 21) by comparison of the structure of the tryptic phosphopeptides with the predicted primary sequence of the EGF receptor reported by Ullrich et al. (22). Phosphopeptide mapping of the EGF receptor isolated from cells treated with PDGF revealed the presence of the same four unique phosphopeptides that were observed in maps of the EGF receptor isolated from PMA-treated cells (Fig. 4). We conclude that PDGF induces the phosphorylation of the EGF receptor at threonine-654.

We quantitated the increase in the phosphorylation state of threonine-654 by resolving the phosphopeptides obtained after trypsin digestion of the EGF receptor by reversed-phase HPLC and measuring the Cerenkov radiation in the eluate using a technique that we have described (16). PDGF (10 ng/ml) was found to be only 16 ± 3% (mean ± standard deviation; $n = 3$) as effective as 10 nM PMA at increasing the phosphorylation state of threonine-654 (not shown). The HPLC procedure demonstrated that PDGF also caused an increase in the phosphorylation state of other phosphopeptides that are present in peptide maps of the EGF receptor isolated from control cells. The major change in the phosphorylation state of the EGF receptor caused by PDGF was observed at a phosphopeptide that contains [^{32}P]phosphothreonine and is the principal site of phosphorylation on the EGF receptor (Fig. 4). This site is also the major site phosphorylated on the EGF receptor after treatment of cells with PMA or EGF (14, 16).

It has been reported that PMA inhibits the tyrosine kinase activity of the EGF receptor in the presence of EGF and that this inhibition can be achieved in vitro by phosphorylation of the EGF receptor at threonine-654 with kinase C (13, 41). As PDGF was observed to cause phosphorylation of the EGF receptor of WI-38 fibroblasts at threonine-654, we investigated the effect of PDGF on the EGF-dependent tyrosine phosphorylation of the EGF receptor (Table 1). Addition of 10 nM EGF to WI-38 fibroblasts caused a 2-fold increase in the phosphorylation state of the EGF receptor (Fig. 2). Phosphopeptide mapping indicated that EGF caused a large increase in the level of a phosphopeptide that was observed to contain phosphotyrosine (Fig. 4). This phosphorylation site has been identified recently as tyrosine-1173 (42). The ability of EGF to induce tyrosine phosphorylation of the EGF receptor in WI-38 cells was inhibited by PMA (Table 1).
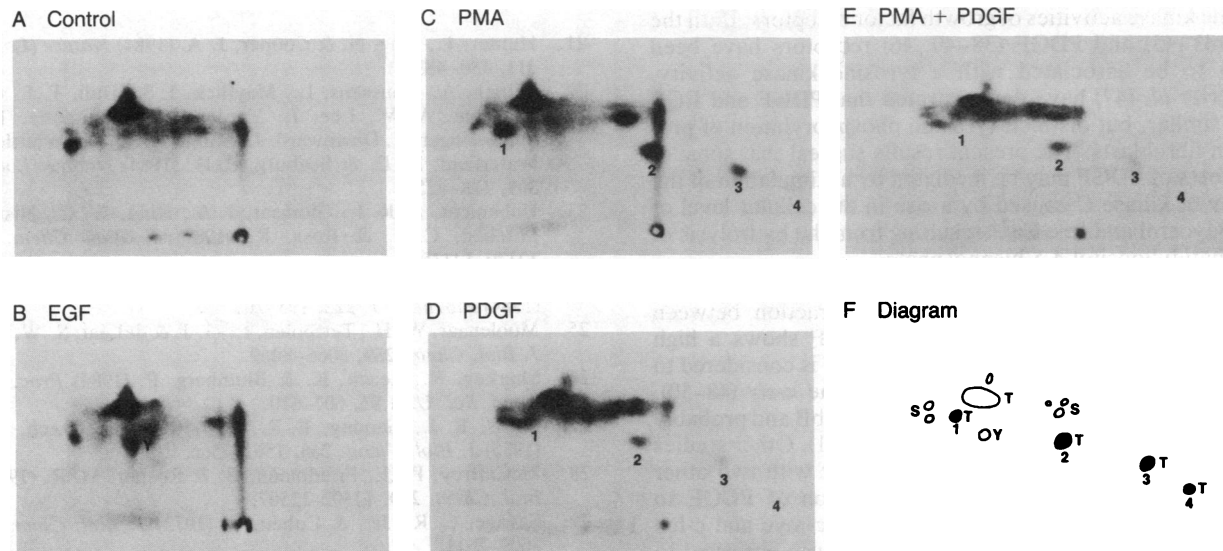
FIG. 4.   Phosphopeptide maps of the EGF receptor isolated from WI-38 fibroblasts. WI-38 fibroblasts in 100-mm dishes were labeled with 15 mCi of [^{32}P]phosphate in 5 ml of medium for 24 hr. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The phosphopeptides obtained from trypsin digestion of the EGF receptors were resolved by electrophoresis and chromatography on 100-μm cellulose-coated thin-layer plates. (A) Control (1052 cpm, Cerenkov radiation; 56-hr exposure during autoradiography). (B) WI-38 cells treated with 10 nM EGF for 10 min (980 cpm; 45 hr). (C) Cells treated with 10 nM PMA for 30 min (1260 cpm; 30 hr). (D) Cells treated with 10 ng of PDGF per ml for 40 min (1066 cpm; 56 hr). (E) The phosphopeptides derived from PMA-treated cells (410 cpm) were mixed with the phosphopeptides obtained from PDGF-treated cells (530 cpm). The autoradiography was for 45 hr. (F) Schematic diagram of the phosphopeptides. Phosphopeptides 1, 2, 3, and 4, which are derived from the incomplete digestion of the sequence surrounding threonine-654, are indicated. The phosphoamino acid composition of the peptides is indicated by S (phosphoserine), T (phosphothreonine), and Y (phosphotyrosine).

Similarly, treatment of the fibroblasts with PDGF also inhibited the EGF-dependent tyrosine phosphorylation of the EGF receptor (Table 1).

DISCUSSION

We report that PDGF causes an increase in the phosphorylation state of the EGF receptor of WI-38 human fetal lung fibroblasts. Phosphopeptide mapping indicates that PDGF increases the phosphorylation state of many sites on the EGF receptor and, in addition, induces the phosphorylation of the

Table 1.   Phosphoamino acid analysis of the EGF receptor isolated from WI-38 fibroblasts

	Phospho-serine, %	Phospho-threonine, %	Phospho-tyrosine, %
Control	70	29	0.1
EGF	141	71	10
PDGF	81	61	0.1
PDGF and EGF	149	69	6
PMA	198	79	0.1
PMA and EGF	203	77	4

WI-38 cells in 100-mm dishes were labeled with 15 mCi [^{32}P]phosphate in 5 ml of medium for 24 hr. The cells were then treated with 10 nM PMA or 10 ng of PDGF per ml for 40 min. EGF (10nM) was subsequently added to some dishes for 5 min. The cells then were solubilized, and the EGF receptors were isolated by immunoprecipitation. The receptors were partially hydrolyzed in 6M HCl, and the phosphoamino acids obtained were. resolved by thin-layer electrophoresis at pH 3.5. Regions of the thin-layer plate corresponding to phosphoamino acids were excised, and the Cerenkov radiation associated with each phosphoamino acid was measured with a β counter. The results are expressed relative to the level of phosphoamino acids recovered from EGF receptors isolated from control cells. 100% represents 2564 cpm. The results presented were obtained in a single experiment. Similar results were observed in two other experiments.

EGF receptor on threonine-654, which is the major site of phosphorylation of the EGF receptor catalyzed by kinase C in vitro (13, 16, 21). This effect of PDGF on the phosphorylation state of the EGF receptor is similar to that observed when cells are treated with PMA (13–16, 21) or exogenous diacylglycerols (27, 28). These results are consistent with the hypothesis that PDGF does stimulate the activity of kinase C in intact fibroblasts. However, one point of caution that must be raised is that the use of the EGF receptor threonine-654 phosphorylation as an indication of the activity of kinase C is invalid if another protein kinase can phosphorylate this site or if a change in phosphatase activity occurs. The ability of PDGF (Fig. 4), PMA (13–16), and diacylglycerol (27, 28) to increase the phosphorylation of the EGF receptor at sites in addition to threonine-654 suggests that these agents might affect the activity of kinases other than kinase C that phosphorylate the EGF receptor. The role of the phosphorylation of the EGF receptor at these sites may be important to the regulation of the receptor. Future studies will be directed towards elucidating the possible roles of these phosphorylation sites.

Addition of PMA or PDGF to intact fibroblasts caused an inhibition of the EGF-dependent tyrosine phosphorylation of the EGF receptor (Table 1). Although measurement of the autophosphorylation of the EGF receptor is not a rigorous method of determining the kinase activity of the EGF receptor, these results suggest that PMA and PDGF cause an inhibition of the tyrosine kinase activity of the receptor. This inhibition of tyrosine kinase activity is consistent with the report that phosphorylation of the EGF receptor on threonine-654 by kinase C in vitro causes an inhibition of the tyrosine kinase activity of the EGF receptor (13). However, the data present the paradox that although PMA and PDGF inhibit the high-affinity binding of ^{125}I-EGF to the EGF receptor (4–12) and inhibit the tyrosine kinase activity of the EGF receptor (13, 41), the mitogenic effects of EGF are enhanced by PMA and PDGF (1–3). This paradox remains to be resolved but does raise interesting questions related to the role of the

tyrosine kinase activities of growth factor receptors. Both the EGF (43–45) and PDGF (38–40, 46) receptors have been shown to be associated with a tyrosine kinase activity. Cooper *et al.* (47) have demonstrated that PDGF and EGF cause similar, but distinct, tyrosine phosphorylation of proteins in fibroblasts. The present results suggest that some of the effects of PDGF may be mediated by a stimulation of the activity of kinase C caused by a rise in the cellular level of diacylglycerol and free Ca^{2+} resulting from the hydrolysis of phosphatidylinositol 4,5-bisphosphate.

The effect of PDGF to cause the phosphorylation of the EGF receptor is an example of the interaction between oncogene products. The B chain of PDGF shows a high degree of sequence homology with v-sis and is considered to be derived from the cellular protooncogene c-sis (48–50). Similarly, the EGF receptor is related to v-erbB and probably represents the product of the c-erbB gene (51). Other studies have shown the ability of PDGF to interact with two other protooncogenes (c-myc and c-fos). Addition of PDGF to fibroblasts rapidly causes the induction of c-myc and c-fos mRNA (52–54). It is of interest that PMA was observed to mimic this action of PDGF (52–54). These results support the concept that cell-growth regulation occurs through the co-ordinate interaction between protooncogene products and that kinase C plays an important role in this process.

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