C. Microscopy

D. Electron microscopy

E. Immunocytochemistry

F. Western blot analysis

G. Immunohistochemistry

H. In situ hybridization

I. Confocal microscopy

J. Laser scanning microscopy

K. Tissue culture

L. Cell culture

M. Cell line

N. Tumor cell line

O. Primary cell culture

P. Cell line derived from tumor

Q. Cell line derived from normal tissue

R. Cell line derived from embryonic stem cells

S. Cell line derived from induced pluripotent stem cells

T. Cell line derived from induced pluripotent stem cells

U. Cell line derived from embryonic stem cells

V. Cell line derived from induced pluripotent stem cells

W. Cell line derived from induced pluripotent stem cells

X. Cell line derived from induced pluripotent stem cells

Y. Cell line derived from induced pluripotent stem cells

Z. Cell line derived from induced pluripotent stem cells

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The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; EGF-stimulated autophosphorylation of the EGF receptor on surface (3-10). In addition, PDGF causes a decrease in the EGF-stimulated autophosphorylation of the EGF receptor on tyrosine residues (9).

Recent studies have demonstrated that some of the actions of PDGF may be mediated by the Ca<sup>2+</sup> /phospholipid-dependent protein kinase (protein kinase C). A rapid effect of PDGF 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 (11, 12). The inositol 1,4,5-trisphosphate causes the release of Ca<sup>2+</sup> from intracellular stores and results in an increased cytosolic free Ca<sup>2+</sup> concentration (12, 13). This dual action of PDGF to increase the level of diacylglycerol and free Ca<sup>2+</sup> would be expected to stimulate the activity of protein kinase C (14).

Evidence that PDGF does activate protein kinase C has been obtained from the analysis of the phosphorylation state of substrate proteins in intact cells. Thus, PDGF causes the phosphorylation of the EGF receptor at threonine 654 (9), calpain I heavy chain at serine 25 (15, 16) and pp60<sup>p56</sup> at serine 12 (17). In addition, PDGF causes the phosphorylation of a less well characterized 80-kDa protein that has been shown to be a protein kinase C substrate (18-20).

The physiological consequences of PDGF action on protein kinase C are not well understood. One possibility is that the activation of protein kinase C by PDGF is the mechanism by which PDGF causes the heterologous regulation of the EGF receptor. Several lines of evidence have been reported that are consistent with this hypothesis. First, PDGF causes the phosphorylation of the EGF receptor at threonine 654, a residue that is the major site of phosphorylation by protein kinase C (9). Second, the regulation of the EGF receptor by PDGF is similar to that observed when cells are treated with tumor-promoting phorbol diesters (21-30) or with synthetic diacylglycerols such as dioctanoylglycerol (31, 32) or oleyl-acetylglycerol (33), (for review see Ref. 34). Third, phosphorylation of the EGF receptor at threonine 654 by protein kinase C in vitro has been shown to inhibit the high affinity binding of 125I-EGF to the receptor (35, 36) and to inhibit the tyrosine kinase activity of the EGF receptor (27, 36). Fourth, the effects of tumor-promoting phorbol diesters in intact cells closely correlate with the phosphorylation state of the EGF receptor at threonine 654 (37, 38). Taken together, these data strongly support the hypothesis that PDGF regulates the EGF receptor by a mechanism that involves the stimulation of protein kinase C and the subsequent phosphorylation of the EGF receptor at threonine 654.
Recently, Olashaw et al. (10) have critically tested the hypothesis that protein kinase C mediates the effect of PDGF to regulate the EGF receptor. The effect of PDGF on control and protein kinase C-deficient BALB/c 3T3 fibroblasts was compared. The method used to deplete the fibroblasts of protein kinase C was to incubate the cells with a high concentration of a tumor-promoting phorbol diester. Collins and Rozengurt (39) have reported that this treatment results in the down-regulation of the binding sites for phorbol diesters and the desensitization of the response of the cells to PMA. The molecular basis of the desensitization has been shown to be due to a loss of protein kinase C activity (40) because of increased proteolysis (41) which results in the complete loss of the 80-kDa form of protein kinase C as determined by Western blotting (20). It would be predicted that if protein kinase C was the mediator of PDGF action, the effects of PDGF should not be observed in fibroblasts that are deficient in protein kinase C. It was observed that PDGF was as potent in protein kinase C-deficient fibroblasts as in control cells. On the basis of these data, Olashaw et al. (10) concluded that protein kinase C is not involved in the mechanism of PDGF action to regulate the EGF receptor.

The purpose of the experiments presented in this report was to investigate the mechanism by which PDGF regulates the EGF receptor in desensitized fibroblasts which are deficient in protein kinase C. We confirm the observations of Olashaw et al. (10), which demonstrate a lack of correlation between protein kinase C expression and PDGF action. We further investigated whether the effects of PDGF on protein kinase C-deficient fibroblasts occurred in the absence of EGF receptor phosphorylation at threonine 654. We show that PDGF causes enhanced phosphorylation of the EGF receptor in cells that are deficient in protein kinase C, indicating that other mechanisms can account for such EGF receptor phosphorylation.

**EXPERIMENTAL PROCEDURES**

Reagents—EGF was prepared as described (42, 43) and iodinated by using the immobilized lactoperoxidase method (44). PDGF purified to homogeneity (45) was a gift from Dr. C. H. Heldin (University of Uppsala). [32P]Phosphate was obtained from Du Pont-New England Nuclear. [γ-32P]ATP and [γ-32P]ATP (50 μCi/ml) were from Amersham Corp. PMA and PBu were purchased from Sigma. The synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg was obtained from Peninsula Laboratories (Belmont, CA). Rat brain protein kinase C was partially purified as described by Kikkawa et al. (46).

Cell Culture—WI-38 human fetal lung 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 treated with 500 nM PBU before use.

**125I-EGF Binding Analysis**—WI-38 fibroblasts grown in 35-mm wells were incubated in Krebs-Ringer HEPES-buffered saline (120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgSO4, 10 mM glucose, 25 mM HEPES, pH 7.4, and 0.2% bovine serum albumin). The cells were treated with PMA or PDGF for 30 min at 37°C and then incubated at 0°C for 4 h with 100 pm 125I-EGF. The monolayers were then carefully washed four times with cold medium and solubilized with 0.1 M NaOH. Radioactivity was quantitated with a Beckman gamma counter. Nonspecific binding was estimated in incubations containing 100 nM EGF.

**Determination of EGF Receptor Phosphorylation State**—WI-38 fibroblasts were labeled with [32P]phosphate for 24 h by incubation with phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 5 μCi of [32P]phosphate per ml. EGF receptors were isolated from the fibroblasts by immunoprecipitation of detergent extracts with a polyclonal anti-EGF receptor antibody as described (29). EGF receptors were then resolved from other components by polyacrylamide gel electrophoresis. Phosphomonoesterase and phosphoprotein extraction were performed by the method of Hunter and Sefton (47) as described (24). Phosphoprotein mapping of the EGF receptor after tryptic digestion (29) was performed by a modification of the HPLC procedure that we have previously described (29). In these experiments a Vydac C8 column (0.4 × 25 cm) equilibrated with 0.1% trifluoroacetic acid was used. Peptides were detected by measuring the Cerenkov radiation associated with a linear gradient of acetonitrile (1% per min) in 0.1% trifluoroacetic acid. Fractions were collected at 30-s intervals, and the [32P]phosphopeptides were detected by measuring the Cerenkov radiation associated with each fraction. The use of a Vydac C8 column in these experiments rather than the Waters bondpak C8 column as previously described (29, 37) enabled the chromatography to be performed without the use of 0.05% triethylamine to modify the mobile phase.

Calcium ion concentration was determined by the fluo-3 method as described (10) and critically tested the hypothesis that the calcium ion concentration increase is required for PDGF to stimulate protein kinase C activity (48).

**Determination of the Phosphorylation of Synthetic Peptides**—WI-38 fibroblasts were washed with Krebs-Ringer HEPES-buffered saline, treated with factors, and then homogenized in 10 mM EGTA, 25 mM HEPES, pH 7.4, 10 μg/ml leupeptin at 4°C by passage 10 times through a tuberculin syringe. In some experiments the homogenate was centrifuged at 100,000 × g for 30 min at 4°C. Kinase assays were performed with 5 μl of cell extract in a final volume of 50 μl containing 25 mM HEPES, pH 7.4, 0.5 mM diithiothreitol, 1 mM MgCl2, 50 μM [γ-32P]ATP (50 μCi/ml), and 0.5 mg/ml synthetic peptide Lys-Arg- Thr-Leu-Arg-Arg. In some experiments 1.5 mM CaCl2 and 25 μg/ml phosphatidylycerine were included in the assay. Phosphorylation of the peptide was observed to be linear with time for up to 20 min. The reaction was terminated by addition of 450 μl of 30% formic acid. The phosphorylation state of the synthetic peptide was determined by spotting 10 μl of the reaction mixture onto a cellulose thin layer plate (0.1 mm × 20 cm × 10 cm, Machery-Nagel) and resolving the phosphorylated components by electrophoresis for 2 h at 400 V in 1% ammonium carbonate. The phosphorylated peptide was detected by autoradiography and quantitated by scraping the cellulose corresponding to the location of the phosphoprotein into a scintillation vial. After the addition of 4 ml of Optiphos (Packard) the radioactivity was determined by scintillation counting.

**Determination of Phosphatase Activity**—In preliminary experiments, the synthetic peptide Lys-Arg- Thr-Leu-Arg-Arg phosphorylated by protein kinase C in vitro and purified by reverse-phase HPLC (29) was used as a substrate for the assay of the phosphatase activity present in cell lysates. However, it was observed that this synthetic peptide was not a substrate for phosphatases. In contrast, the intact EGF receptor phosphorylated at threonine 654 is a substrate for phosphatases (27). We therefore utilized EGF receptor purified from A431 membranes specifically phosphorylated with [32P]phosphate at threonine 654 as the substrate for these experiments.

EGF receptors were purified from A431 membranes by affinity chromatography as described (50). Autophosphorylation of the EGF receptor was inhibited by 2 mM diethiothreitol (29). The inactivated EGF receptors were then incubated in 0.2% Triton X-100, 10 mM MgCl2, 1 mM CaCl2, 50 μg/ml phosphatidylycerine, 25 mM HEPES, pH 7.4, 500 μM [γ-32P]ATP (10 μCi/nmol), and 500 micromolar (1 unit = 1 μmol/min) partially purified rat brain protein kinase C. The incubation was terminated by the addition of 2 mM glucose and 1 milliunit of hexokinase. The phosphorylation state of the EGF receptors was then examined by immunoprecipitating the receptors with a polyclonal antibody, polypeptide gel electrophoresis, and phosphoprotein mapping as described (29). It was observed that the major site of phosphorylation on the EGF receptor under these conditions is threonine 654 (data not shown). This specifically phosphorylated EGF receptor preparation was used as the substrate for phosphatase assays.

Phosphatase assays were performed by using the cells prepared by passage of the cells 10 times through a tuberculin syringe in 10 mM EGTA, 10 μg/ml leupeptin, and 25 mM HEPES, pH 7.4.
RESULTS

In order for WI-38 fibroblasts to be a useful model system for the present studies it was necessary to demonstrate in these cells the loss of protein kinase C activity that has been reported for other cultured cell lines after prolonged incubation with phorbol diesters. Protein kinase C activity could be readily monitored in cell extracts in the presence of added Ca" and phosphorylserine using the peptide Lys-Arg-Thr-Leu-Arg-Arg as substrate (Fig. 1). WI-38 fibroblasts were found to possess a high level of protein kinase C activity which is not detectable in cells that had been incubated for 48 h with 500 nM phorbol dibutyrate (PBU). We then examined whether this loss of protein kinase C activity was associated with the desensitization of the fibroblasts to the actions of phorbol diesters. PMA has been reported to inhibit the high affinity binding of 125I-EGF to WI-38 fibroblasts (9, 29). Scatchard analysis of 125I-EGF binding to fibroblasts yields a curvilinear plot suggesting the presence of high affinity and low affinity receptor sites (Fig. 2). Fig. 2 also demonstrates that fibroblasts incubated for 48 h with phorbol dibutyrate are desensitized to the action of PMA to inhibit 125I-EGF binding to the high affinity sites. This result is consistent with the loss of the putative receptor for phorbol diesters, protein kinase C (Fig. 1).

PDGF Regulates the Apparent Affinity of the EGF Receptor

PDGF Causes Phosphorylation of the EGF Receptor in Protein Kinase C-deficient WI-38 Fibroblasts—We examined whether the loss of protein kinase C activity and the desensitization of WI-38 fibroblasts to phorbol diesters affected the response of the cells to PDGF. It was observed that PDGF inhibited the high affinity binding of 125I-EGF to WI-38 fibroblasts that had been desensitized with phorbol diester and that this effect of PDGF was similar to that observed in experiments using control fibroblasts (Fig. 2). Low affinity binding of 125I-EGF to the cells was not modulated by PDGF. Similar results have been reported by Olashaw et al. (10) in experiments with BALB/c/3T3 fibroblasts.
EGF receptor phosphorylation state following PDGF treatment was 1.8 ± 0.3-fold and 1.2 ± 0.1-fold (n = 4) for control and desensitized fibroblasts, respectively. In contrast, EGF treatment caused a marked increase in the phosphorylation state of the EGF receptor in desensitized as well as control fibroblasts (3.6 ± 0.4-fold and 3.1 ± 0.5-fold (n = 4) in control and desensitized fibroblasts, respectively).

In further experiments the phosphorylation state of the EGF receptor was examined in detail (Fig. 4). Phosphoamino acid analysis of EGF receptors isolated from control and desensitized 32P-labeled cells demonstrated similar levels of [32P]phosphoserine and [32P]phosphothreonine. Addition of PMA to control fibroblasts increased serine and threonine phosphorylations on EGF receptors, whereas in desensitized cells no effect of PMA was observed. Increased levels of EGF receptor [32P]phosphothreonine were observed in the desensitized cells compared with control cells (Fig. 4).

Tryptic phosphopeptide mapping was performed to analyze the sites phosphorylated on the EGF receptor in response to EGF, PMA, or PDGF (Fig. 5). Although similar levels of phosphorylation of many peptides were observed when HPLC tryptic peptide maps of EGF receptors isolated from control and desensitized cells were compared, a marked difference was found in the level of a phosphopeptide that eluted from the reverse-phase column at 7% acetonitrile (Fig. 5). We have previously identified this phosphopeptide as Lys-Arg-Thr(P)-Leu-Arg (29). The phosphorylated residue corresponds to EGF receptor threonine 654 and is the major site on the EGF receptor that is phosphorylated by protein kinase C (28, 29).

We calculate that the stoichiometry of phosphorylation of threonine 654 in control fibroblasts is 0.06 ± 0.02 (mean ± standard deviation; n = 3) mol of phosphate per mol of receptor. In desensitized fibroblasts the stoichiometry is 0.16 ± 0.02 (Fig. 6). Thus, WI-38 fibroblasts desensitized with phorbol dibutyrate which lack detectable protein kinase C activity (Fig. 1) possess EGF receptors that are phosphorylated to a greater extent at threonine 654 than control cells (Fig. 6), in which a high level of protein kinase C activity is observed (Fig. 1).

EGF was observed to cause a similar increase in the phosphorylation state of the EGF receptor in control and desensitized fibroblasts (Fig. 3). However, phosphoamino acid analysis indicated that, although similar levels of [32P]phosphoserine and [32P]phosphothreonine were observed, a significant decrease in the level of [32P]phosphothreonine was observed in the EGF receptors isolated from desensitized fibroblasts treated with EGF compared with control cells treated with EGF (Fig. 4). This result was confirmed by HPLC tryptic phosphopeptide mapping (Fig. 5). A phosphopeptide containing the major site of autophosphorylation (tyrosine 1173; Ref 51) elutes from the reverse-phase column at 22% acetonitrile. A decreased level of this phosphopeptide was observed in EGF receptors isolated from EGF-treated, desensitized fibroblasts compared with EGF-treated control cells. A decrease in the EGF-stimulated tyrosine phosphorylation of the EGF receptor in desensitized fibroblasts is consistent with the observation of significant phosphorylation of EGF receptors at threonine 654 in these cells (Figs. 5 and 6). It has previously been shown that phosphorylation of the EGF receptor at threonine 654 (28, 29) correlates with an inhibition of the apparent tyrosine protein kinase activity of the receptor (25, 27, 31).

The sites on the EGF receptor phosphorylated in the presence of PDGF include a major phosphothreonine-containing peptide (that elutes from the HPLC column at 28% acetonitrile) and two closely resolved peptides containing [32P]phosphoserine (that elute from the HPLC column at 34% acetonitrile) in addition to threonine 654. A similar pattern of phosphorylation is observed when cells are treated with PMA (24–30) or with a synthetic diacylglycerol (31–33). In phorbol diester–desensitized fibroblasts the effects of PDGF are completely inhibited but the effects of PDGF are only partially inhibited (Fig. 3). The regulation of the phosphorylation state of EGF receptor phosphorylation sites in phorbol diester–desensitized fibroblasts was therefore examined. The results demonstrate that, in the presence of PDGF, the same extent of phosphorylation of threonine 654 is observed in desensitized fibroblasts and in control fibroblasts (0.2 ± 0.04 and 0.21 ± 0.03 (mean ± standard deviation, n = 3) mol of phosphate per mol of receptor after 15 min of PDGF treatment for control and desensitized fibroblasts, respectively; Fig. 6). However, the percent increment in threonine 654 phosphorylation caused by PDGF in desensitized cells was less than that observed in control cells because of the higher basal level of phosphorylation of this residue in desensitized cells (0.06 ± 0.02 and 0.16 ± 0.02 (mean ± standard deviation, n = 3) mol of phosphate per mol of receptor for control and desensitized fibroblasts respectively; Fig. 6).

In contrast to the results obtained from the analysis of the stoichiometry of phosphorylation of EGF receptor threonine 654, the effect of PDGF to cause phosphorylation of other sites on the EGF receptor was inhibited in phorbol diester–desensitized fibroblasts. Since PDGF inhibits the high affinity binding of EGF to desensitized fibroblasts, we conclude that the phosphorylation state of the EGF receptor at sites other than threonine 654 does not correlate with the apparent affinity of the receptor. A similar conclusion has been reached for the effect of PMA to regulate the apparent affinity of EGF receptors (37).

**Comparison of the Effect of Phorbol Diester and PDGF to Inhibit the High Affinity Binding of 125I-EGF to Human Fibroblasts and to Cause Phosphorylation of the EGF Receptor at Threonine 654**—In order to test the hypothesis that phosphorylation of the EGF receptor at threonine 654 is the mechanism by which PDGF regulates the apparent affinity of the EGF receptor, we compared in detail the magnitude of the effects of PDGF with those of the tumor-promoting phor-
FIG. 5. Phosphopeptide maps of EGF receptor isolated from WI-38 fibroblasts. EGF receptors were isolated from [32P]phosphate-labeled WI-38 fibroblasts by immunoprecipitation and polyacrylamide gel electrophoresis. The isolated receptors were then incubated with trypsin and the [32P]phosphopeptides obtained were resolved by reverse-phase HPLC. The [32P]phosphopeptides were detected by measuring the Cerenkov radiation associated with each fraction collected. The HPLC elution profiles obtained from cells treated without (A) and with 10 nM PMA (B–D), 10 nM EGF (E–G), or 0.7 nM PDGF (H–J) are presented. The profiles obtained from control cells and desensitized cells are presented by solid and dashed lines, respectively. The [32P]phosphopeptides corresponding to threonine 654 and tyrosine 1173 are marked in panels B and E. Similar results were obtained in three separate experiments.
Regulation of Kinase and Phosphatase Activities Specific for EGF Receptor Threonine 654 in WI-38 Fibroblasts—Phosphopeptide mapping of the EGF receptor from control and desensitized WI-38 fibroblasts demonstrated that a higher level of phosphorylation of threonine 654 was observed in desensitized cells (Figs. 5 and 6). This increase in phosphorylation state could be caused by either the increase in the activity of a protein kinase or a decrease in the activity of a protein phosphatase. We therefore investigated the activities of protein kinases and phosphatases specific for EGF receptor threonine 654 in extracts of WI-38 fibroblasts. No significant difference was found between the phosphatase activity observed in lysates prepared from control and desensitized fibroblasts (Fig. 9). Furthermore, no difference in lysate phosphatase activity was found between cells treated with and without 10 nM EGF or 0.7 nM PDGF for 5 min at 37 °C (data not shown).

In contrast to the lack of detectable effects on phosphatase activity, an increased kinase activity was observed in lysates
FIG. 8. Correlation between the phosphorylation state and the apparent affinity of the EGF receptor. WI-38 fibroblasts were incubated without and with different concentrations of PDGF (●) and PMA (■) for 15 min. The binding of 100 pm ¹²⁵I-EGF to cell surface receptors was then determined (A). The stoichiometry of phosphorylation of the EGF receptor at threonine 654 was calculated from the data presented in Fig. 7 as described in the legend to Fig. 6 (B). A comparison of the effects of PDGF and PMA to cause phosphorylation of the EGF receptor at threonine 654 and to inhibit the high affinity binding of ¹²⁵I-EGF to cell surface receptors is presented in C. The results represent the mean of three separate experiments.

of desensitized fibroblasts compared with those of control cells (Fig. 10). The desensitized fibroblasts lack protein kinase C as demonstrated by the measurement of the phosphorylation of the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg by cytosolic cell extracts in a Ca²⁺- and phospholipid-dependent manner (Fig. 1). However, when total fibroblast homogenates were assayed in the absence of Ca²⁺, an increased kinase activity was present in extracts prepared from desensitized cells compared with control cells (Fig. 10). Centrifugation of the homogenates removed the kinase activity from the cell extracts, indicating that the kinase is probably bound to membranes (Table I). The kinase activity could be shown to be distinct from protein kinase C by the demonstration that it is inhibited by Ca²⁺ under conditions where protein kinase C is stimulated (Fig. 10). The effect of treatment of the fibroblasts with EGF and PDGF on this kinase activity was examined. However, no consistent effect of growth factor treatment was observed under these conditions on the activity of protein kinases that phosphorylate the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg assayed in vitro.

DISCUSSION

Many effects of PDGF are similar when examined in control fibroblasts and protein kinase C-deficient fibroblasts desensitized with phorbol diester. Examples include the incorporation of [³H]thymidine into acid-insoluble material, increased glucose transport, increased cytosolic Ca²⁺ concentration, increased tyrosine phosphorylation, and inhibition of the high affinity binding of EGF (10, 39, 52-55). It has been concluded from these studies that protein kinase C is not required for these effects of PDGF. In contrast, other actions of PDGF such as the induction of c-myc and ornithine decarboxylase are attenuated in desensitized fibroblasts (53, 56).
TABLE I

<table>
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<tr>
<th>Subcellular localization of protein kinase activity</th>
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<td>WI-38 fibroblasts were incubated with and without 500 nM PBU for 48 h. The cells were then washed with serum-free medium and subsequently homogenized. In some assays the homogenates were centrifuged at 100,000 × g for 30 min to yield a supernatant fraction that was used for investigation of kinase activity. The kinase activity present in these cell extracts was assayed using the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg as a substrate. In some assays 25 μg/ml phosphatidylserine or CaCl₂ (500 μM in excess over the concentration of EGTA) was added to the assay. The products of the reaction were analyzed by thin layer electrophoresis and autoradiography. The results were quantitated by measuring the Cerenkov radiation associated with the 32P-phosphopeptide. Similar results were obtained in three separate experiments.</td>
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<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Addition to assay</th>
<th>Control</th>
<th>Ca²⁺</th>
<th>Phosphatidylerine</th>
<th>Ca²⁺ and Phosphatidylerine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>198</td>
<td>214</td>
<td>370</td>
<td>1906</td>
<td></td>
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<tr>
<td>Supernatant</td>
<td>168</td>
<td>289</td>
<td>350</td>
<td>2078</td>
<td></td>
</tr>
<tr>
<td>Pretreated with PBU</td>
<td>590</td>
<td>260</td>
<td>574</td>
<td>275</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>140</td>
<td>148</td>
<td>143</td>
<td>138</td>
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From these experiments it has been concluded that PDGF can increase the expression of these genes by both a protein kinase C-independent and a protein kinase C-linked pathway. The regulation of EGF receptor affinity by PDGF is an example of an action of PDGF that is not altered by desensitization of fibroblasts to phorbol diesters (10). The effects of PDGF to regulate the EGF receptor may therefore be independent of protein kinase C. The availability of desensitized fibroblasts that are deficient in protein kinase C allowed us to test further the hypothesis that PDGF action to regulate the EGF receptor is mediated by increased phosphorylation of the EGF receptor at threonine 654. It was observed that PDGF inhibited the apparent affinity of the EGF receptor and caused phosphorylation of the EGF receptor at threonine 654 to a similar extent in control and protein kinase C-deficient cells (Figs. 2 and 5). This result indicates that the expression of protein kinase C by cells is insufficient to account for the increased phosphorylation of the EGF receptor at threonine 654 caused by PDGF and that other protein kinases or protein phosphatases may be involved.

An unexpected observation that we report is that fibroblasts desensitized to phorbol diesters which lack detectable protein kinase C activity (Fig. 1) demonstrate increased phosphorylation of a protein kinase C substrate, EGF receptor threonine 654 (Fig. 5). This result suggests that desensitized fibroblasts either have an increased activity of another kinase or decreased activity of a phosphatase. No evidence for a significant change in phosphatase activity was obtained (Fig. 9), but a significant increase in the activity of a membrane-bound Ca²⁺-inhibited protein kinase was detected using the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg as a substrate (Fig. 10, Table I). Functional significance of the increased phosphorylation of the EGF receptor at threonine 654 is demonstrated by the decreased EGF-stimulated autophosphorylation of the receptor on tyrosine residues (Fig. 4). It has previously been shown that phosphorylation of the EGF receptor at threonine 654 in vitro causes an inhibition of the tyrosine kinase activity of the EGF receptor (27).

Previous studies have investigated the phosphorylation state of an 80-kDa endogenous substrate for protein kinase C in protein kinase C-deficient fibroblasts. Rozengurt et al. (18) reported that the desensitization of Swiss 3T3 cells to phorbol diesters does not alter the incorporation of [32P]phosphate into the 80-kDa protein. Blackshear et al. (20) observed that desensitization of 3T3-L1 fibroblasts caused a large increase in the incorporation of [32P]phosphate into the 80-kDa protein. In contrast, Coughlin et al. (53) reported that no incorporation of [32P]phosphate into the 80-kDa protein was observed when human fibroblasts were desensitized. The variable results reported by these groups may be due to an alteration in the level of expression of the 80-kDa protein in fibroblasts exposed to phorbol diesters so that a comparison of total [32P]phosphate incorporation does not provide a reliable indication of the stoichiometry of phosphorylation. Furthermore, the analysis of the total incorporation of [32P]phosphate into the 80-kDa protein does not provide a reliable indication of protein kinase C phosphorylation if multiple sites on this protein are phosphorylated by other protein kinases.

The increased activity of a membrane-bound Ca²⁺-inhibited protein kinase observed after desensitization of WI-38 fibroblasts with phorbol diesters warrants further investigation. The identity of this kinase is not known, but it appears to be functionally distinct from protein kinase C, which is activated in the presence of Ca²⁺. This protein kinase activity may be due to the induction of a novel enzyme or may be due to the presence of a modified form of protein kinase C in desensitized fibroblasts. Cochet et al. (57, 58) have reported that phorbol diesters cause protein kinase C to bind to membranes and result in an alteration in the catalytic properties of the enzyme. One example of a modification of protein kinase C that might be caused by phorbol diesters is the generation of an active proteolytic fragment. Protein kinase M is a soluble enzyme and is therefore unlikely as a candidate for the membrane-bound kinase observed in desensitized cells. However, the immunoreactive 67-kDa protein kinase C-related protein described by Girard et al. (59, 60), which is found as a membrane protein in brain (59, 60), is a possible candidate. Important future objectives for further progress in this field are the purification of the membrane-bound kinase activity observed in desensitized fibroblasts, its detailed characterization, and the investigation of the role of this protein kinase in the growth factor-stimulated protein phosphorylation.

The observation (Fig. 10, Table I) of a protein kinase activity in phorbol diester-desensitized fibroblasts may account for the long-term effects of phorbol diesters which persist after the apparent desensitization of the cells. Examples include elevated glucose transport, cell surface fibronectin, and alterations in cell morphology (61, 62). These persistent actions of phorbol diesters on desensitized cells are an important consideration for the interpretation of experiments designed to investigate the role of protein kinase C-linked phosphorylation in growth factor action (e.g. Refs. 10, 52–56). Experiments on the human fibroblast EGF receptor (Fig. 6) indicate that the down-regulation of protein kinase C does not necessarily lead to the inhibition of growth factor-stimulated protein phosphorylation. These data demonstrate that caution needs to be exercised in the interpretation of experiments in which the ability of phorbol diesters to cause heterologous desensitization of growth factor action is investigated. In contrast to the effects of desensitization with phorbol

The protein kinase activity observed is inhibited by Ca²⁺. However, as the assays were performed using crude cellular extracts, it is not known whether the inhibition is because of a direct inhibitory action of Ca²⁺ on the enzyme or whether the inhibition is due to an indirect action of Ca²⁺ such as the stimulation of proteases. However, we note that the assays were performed in the presence of 10 μg/ml leupeptin and that, under identical conditions, protein kinase C is stimulated by Ca²⁺.
phorylation and apparent affinity (Figs. 4 and 7). The observation of EGF receptor phosphorylation at threonine 654 in desensitized fibroblasts (Fig. 2) and the lack of quantitative correlation between receptor phosphorylation and apparent affinity (Fig. 8) may be due to a difference between the average stoichiometry of phosphorylation of the total receptor population versus the stoichiometry of phosphorylation of the relevant high affinity receptors. This hypothesis predicts that the reason why PDGF inhibits the binding of EGF to cells at a lower stoichiometry of receptor phosphorylation than PMA is because PDGF is able to specifically cause phosphorylation of the high affinity class of EGF receptors, whereas PMA causes phosphorylation of the total pool of EGF receptors. This hypothesis is currently being tested.

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Epidermal Growth Factor Receptor Regulation