Journal of Cellular Biochemistry 34:1–11 (1987)
Growth Factors, Tumor Promoters, and Cancer Genes 89–99

Growth Factors Modify the Epidermal Growth Factor Receptor Through Multiple Pathways

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Previous results have shown that tumor promoters modify the properties of the epidermal growth factor (EGF) receptor through the activation of protein kinase C. Diacylglycerol-generating factors such as platelet-derived growth factor (PDGF) and $p28^{sis}$ should activate protein kinase C and alter EGF receptor properties in a similar manner. To test directly the involvement of protein kinase C in the action of media from v-*sis*–transformed cells on the EGF receptor, Swiss 3T3 cells were first extensively treated with various concentrations of the tumor-promoter phorbol dibutyrate (PDBu) This treatement reduced levels of active protein kinase C in the cells, making them less responsive to subsequent rechallenge with the tumor promoter. The results demonstrate that there are at least two components to the action of media from v-*sis* transformed cells on EGF binding: a labile factor that confers protein kinase C independence and a stable factor that appears to be dependent on protein kinase C. The action of the first factor cannot be mimicked by transforming growth factor-β or EGF in either the presence or absence of PDGF. The action of the second factor is similar to that of PDGF. These findings indicate that heterologous regulation of the EGF receptor can occur through both protein kinase C-dependent and -independent pathways.

Key words: regulation, multiple pathways, EGF receptor

In previous work, we [1,2] and others [3–6] have shown that tumor promoters modulate the action of the epidermal growth factor (EGF) receptor. Three effects are observed in human cell lines: 1) reduction of EGF binding to the apparent high affinity EGF receptor, 2) decrease in EGF-stimulated tyrosine kinase activity as monitored by EGF receptor tyrosine phosphorylation, and 3) increase in overall EGF receptor phosphorylation at serine and threonine residues. Since these effects appear to be mediated by protein kinase C, growth factors that can generate diacylglycerol, the endogenous activator of protein kinase C [7], should modify the EGF receptor in a similar manner. Platelet-derived growth factor (PDGF), which generates diacylglycerol through phosphatidylinositol turnover, falls into this category [8–11]. Simian

Received June 6, 1986; revised and accepted December 15, 1986.

sarcoma virus (v-*sis*)-transformed NRK cells contain $p28^{sis}$, a PDGF-like protein that is the product of the v-*sis* oncogene. To investigate directly the role of protein kinase C in mediating changes in EGF-receptor properties, we determined whether conditioned medium from v-*sis*-transformed cells was capable of modifying EGF binding to the EGF receptor in protein kinase C-depleted Swiss 3T3 cells.

The results indicate that 1) there is an alternative pathway to protein kinase C for heterologous regulation of the EGF receptor; 2) this alternative pathway must arise, at least in part, from a component other than PDGF-like factors; and 3) since medium from v-*sis*–transformed cells does not act on cellular regulatory pathways solely through the PDGF receptor, some of the growth-promoting and -transforming properties ascribed to the product of the v-*sis* gene may in fact be due to the action of other factors.

MATERIALS AND METHODS

Materials

Mouse EGF (Collaborative Research, Inc., Lexington, MA) was iodinated by the chloramine T method to a specific activity of approximately 6 Ci/μmol using NA-^{125}I (Amersham, Arlington Heights, IL). Phorbol diterpene esters were purchased from Sigma (St. Louis, MO). Medium from v-*sis*–transformed NRK cells was obtained from the laboratory of C. Stiles. It was concentrated using a Millipore PT6C series membrane (MW cutoff of 10,000 Da) from serum-free DME/F12 (1:1) that had been conditioned by confluent cultures of v-*sis*–transformed NRK cells for 2 days. One unit of activity is defined as the amount of medium that induces 50% DNA synthesis in a quiescent monolayer of BALB/c-3T3 cells. Purified porcine PDGF was obtained from BRL and shown to be free of TGF-β by R. Assoian.

Cultures

Swiss 3T3 cells were grown in a gassed (5.5% CO_2), humidified incubator in Dulbecco's modified Eagle's medium (DME) supplemented with 10% heat-inactivated fetal calf serum. When the cells reached confluence, the medium was removed and replaced with DME containing 0.5% bovine serum albumin (BSA) for 48 hr. To generate cells having different levels of protein kinase C, cultures were treated for an additional 48 hr with 0, 10, 100, or 1,000 ng/ml phorbol dibutyrate (PDBu) in DME/0.5% BSA.

Quantitation of ^{125}I-EGF Binding to High Affinity EGF Receptors

Confluent, quiescent cells, grown on 24 well dishes, were treated with various concentrations of PDBu for 48 hr as described above. The cultures were then washed three times with binding medium over a period of 2 hr at 37°C. This medium was removed and appropriate agents added in a total volume of 0.2 ml binding medium for the times indicated in the figure legends. Each variable was tested in triplicate. Cells were then placed on ice and washed with binding medium. ^{125}I-EGF (0.05–0.1 nM) in binding medium was added for 4–6 hr at 4°C. This concentration of ^{125}I-EGF binds primarily to high affinity EGF receptors, as demonstrated by the 80% reduction in cpm bound resulting from 37°C PDBu treatment of cells that had not been depleted of protein kinase C. Cells were washed, lysed, and quantitated for specific ^{125}I-EGF binding as described. Data were normalized to the amount of specific ^{125}I-EGF

binding to cells that were treated with binding medium alone. Since treatment with PDBu elicits a round of mitotic division, both cell and receptor numbers vary in the different populations. Thus, given the different absolute numbers, the data must be expressed as percentages. In general, the cpm bound ranged from 1,000 to 4,000 cpm, with a standard deviation of 5–10%. The nonspecific EGF binding was 100 cpm. The specifics for each experiment presented here are given in the figure legends.

Preparation of Cell Extracts for Assay of Protein Kinase C

150 cc plates of confluent, quiescent Swiss 3T3 cells, which had been treated with 0, 10, 100, or 1,000 ng PDBu/ml for 48 hr at 37°C, 5.5% CO_2, were placed on ice and washed three times with phosphate-buffered saline. Cells were then lysed in 1 ml of 20 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 5 mM dithiothreonine (DTT), 1 mM PMSF, 10 μg approtinin/ml, and 1% Triton-X 100, pH 7.4. Plates were then washed with 0.5 ml lysis buffer, and the lysate and wash were pooled. After centrifugation for 60 min at 35,000 rpm, the supernatants were loaded onto 1 ml DEAE 52 cellulose columns. These columns were washed extensively with 20 mM Tris, 2 mM EDTA, 0.5 mM EGTA, and 1 mM DTT, pH 7.4, before the samples were applied and with 50 ml of the same buffer after samples were loaded. Columns were eluted with a 40 ml continual salt gradient ranging from 0 to .15 M NaCl, and 1 ml fractions were collected. Samples were at all times kept on ice or at 4°C.

Protein Kinase C Assays of Column Fractions

Fifty lambda of each fraction with 50 lambda reaction mix was assayed for protein kinase C activity at 30°C for 10 min. The reaction mixture contained 20 mM Tris, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.8 mg histones/ml, 0.1 mM ATP, 2 μCi γ-^{32}P-ATP, 0.32 mg phosphatidyl serine/ml, and 400 ng PDBu/ml, pH 7.4. In control samples, the phosphatidylserine and PDBu were omitted. The reaction was started by addition of the reaction mixture and terminated by spotting 90 lambda of the reaction on phosphocellulose papers. The papers were washed extensively with 30 mM phosphoric acid, dried, and counted for histone-associated radioactivity. The activity of the pooled peak fractions was determined as described above except that, for these determinations, aliquots of 10, 20, 30, 40, and 50 lambda were assayed in triplicate.

Protein Assays

Protein concentration of cell lysates and pooled peak fractions were determined by absorbance at 595 μm using Bio-Rad protein reagent and BSA as a standard.

RESULTS

To determine whether the medium from v-*sis*–transformed NRK cells could modify EGF receptors in murine Swiss 3T3 cells, changes in EGF receptor binding were monitored. Cells were initially treated at 37°C for 60 min with either PDBu (100 ng/ml), which activates protein kinase C, or v-*sis*–medium (100 units/ml). These cells were then incubated at 4°C with ^{125}I-EGF and cell-associated radioactivity determined. Scatchard analysis revealed two populations of EGF receptors in untreated cells with K_d values of approximately 4×10^{-11} M and 1×10^{-9} M corresponding to approximmately 3×10^2 and 2×10^4 receptor molecules per cell, respectively (Fig. 1). Treatment of cells with either medium from v-*sis*–transformed

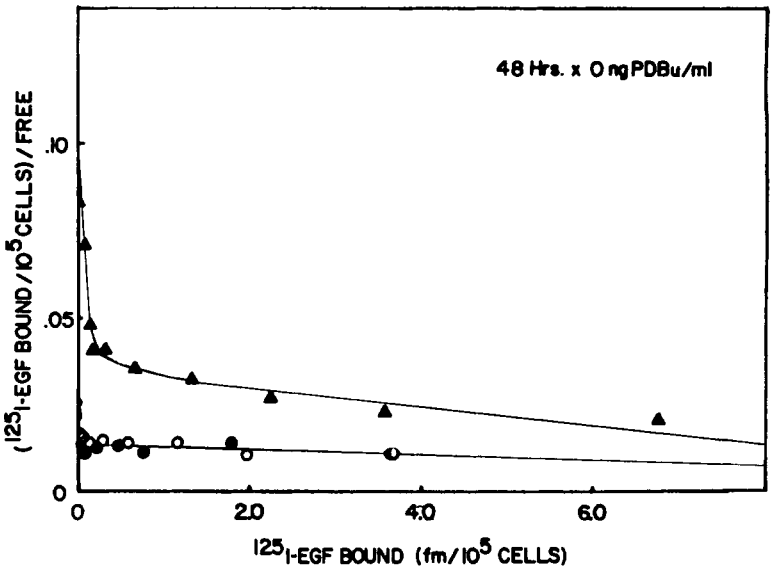

Fig. 1. Scatchard analysis of 4°C ^{125}I-EGF binding to Swiss 3T3 cells. Confluent quiescent Swiss 3T3 cells were treated at 37°C for 15 min with control medium (▲), PDBu (100 ng/ml; ○), or medium from v-*sis*-transformed cells (100 units/ml; ●). These cells were then assayed for 4°C ^{125}I-EGF binding as described in Materials and Methods. Analysis of two independent experiments gives high affinity K_d values of 3.7×10^{-11} and 2.1×10^{-11}, and low affinity K_d values of 1.3×10^{-8} and 0.91×10^{-8}, respectively.

cells or PDBu inhibited EGF binding to the high affinity receptor without significantly affecting low affinity EGF binding.

To determine whether the action of this medium on the EGF receptor is dependent on protein kinase C, we investigated the effect of the medium from v-*sis*-transformed NRK cells on EGF binding in cells depleted of protein kinase C. Confluent quiescent Swiss 3T3 cells were depleted of protein kinase C to various extents by exposure to 0, 10, 100, or 1,000 ng/ml PDBu for 48 hr. We assayed the protein kinase C activity in these cells by determining the amount of Ca^{2+} phospholipid-activated kinase actvity in DEAE cellulose fractions of cell extracts. The results indicate that this treatment depletes cells of protein kinase C in a dose-dependent manner (Fig. 2).

Changing cellular levels of protein kinase C did not significantly affect the ability of the media from v-*sis*-transformed cells to inhibit high affinity EGF binding. This lack of dependence of the medium on protein kinase C was observed over a 2 hr time course (Fig. 3) and a 100-fold range in concentration independent of dose (Fig. 4A). In contrast, depletion of protein kinase C in these cells dramatically reduced the ability of PDBu to inhibit high affinity EGF binding over a 100-fold range in concentration (Fig. 4B). These experiments demonstrate that the medium from v-*sis*-transformed cells modulates EGF receptor properties in a completely protein kinase C-independent manner.

To determine whether the PDGF-like factors in the medium from v-*sis*-transformed cells could be responsible for this effect, the action of PDGF was examined. PDGF (Fig. 4C) caused a rapid, dose-dependent loss of high affinity EGF binding

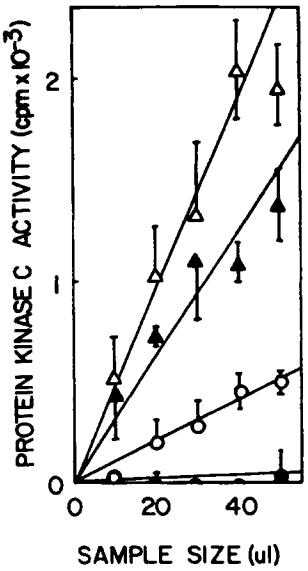

Fig. 2. Protein kinase C activity in pooled peak DEAE column fractions. Peak fractions of protein kinase C activity were pooled and assayed to determine the specific activity of protein kinase C following 48 hr treatment of confluent quiescent cells with 0 (△), 10 (▲), 100 (○), or 1,000 (●) ng/ml PDBu. Each point represents the mean of triplicate determinations of protein kinase C activity from which Ca^{2+} phospholipid-independent kinase activity was subtracted. Standard deviations are indicated by bars.

(80%) in untreated Swiss 3T3 cells. This loss of high affinity EGF binding appeared to be dependent on protein kinase C, although some activity in the protein kinase C-depleted cells was noted. In cells with depleted levels of protein kinase C, PDGF treatment resulted in less than 50% inhibition of EGF receptor binding. These results indicate that PDGF cannot account for the protein kinase C-independent inhibition of high affinity EGF binding by the media from v-*sis*–transformed cells.

The action of PDGF is strikingly similar to that in the medium from v-*sis*–transformed cells following freeze-thaw treatment. After several cycles of freeze-thawing (Fig. 5A) or heat treatment at 100°C for 5 min (data not shown), the medium still caused a rapid, dose-dependent loss of high affinity EGF binding in normal Swiss 3T3 cells that was maintained for 2 hr. However, neither the freeze-thawed medium (Fig. 5A), PDGF (Fig. 5B), nor PDBu (Fig. 5C) was able to inhibit completely the EGF binding in the C kinase-deficient cells over the same time course. These results suggest that at least one component of the media from v-*sis*–transformed cells required for total protein kinase C-independent inhibition of EGF binding may not be related to the PDGF-like peptides.

To identify other potential components in the medium from v-*sis*–transformed cells that might mediate inhibition of EGF binding, we examined the action of transforming growth factor-β (TGF-β) and EGF. TGF-β is secreted into the medium of transformed cells, is labile to heat treatment under certain conditions, and blocks high affinity EGF binding in NRK cells [12,13]. However, TGF-β had little effect on high affinity EGF binding in Swiss 3T3 cells (Fig. 6A) and did not significantly increase the inhibition of EGF binding by PDGF in protein kinase C-depleted cells (Fig. 6B,C). TGF-α, an EGF-like factor, is also secreted into the medium of a

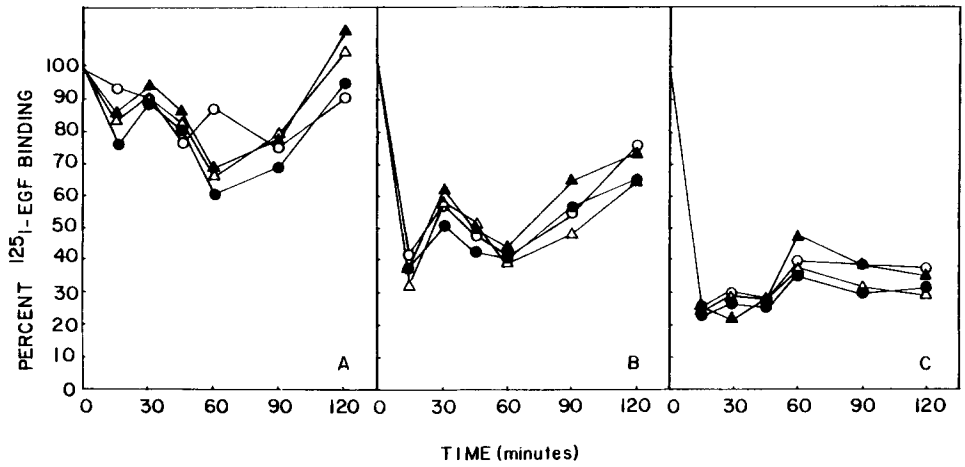

Fig. 3. Effect of protein kinase C depletion on the time course of action of medium from v-*sis*-transformed cells in blocking high affinity ^{125}I-EGF binding to Swiss 3T3 cells. Confluent quiescent Swiss 3T3 cells were treated with 0 (●), 10 (○), 100 (▲), 1,000 (△) ng/ml PDBu for 48 hr. They were then exposed at 37°C to **A)** 1 unit/ml, **B)** 10 units/ml, or **C)** 100 units/ml of medium from v-*sis*-transformed cells for times ranging from 15 min to 2 hr. The data are expressed as the percentages of ^{125}I-EGF binding in cells treated with either PDBu or medium from v-*sis*-transformed cells relative to untreated control cells. The specific binding of ^{125}I-EGF constituting 100% for control and PDBu-pretreated cells ranged from 700 to 1,100 cpm. Nonspecific binding was approximately 100 cpm. Each point represents the mean of triplicate samples. Standard deviations were less than 10%. The concentration of ^{125}I-EGF used in these studies was 0.05 nM.

number of cells and competes with EGF for binding to the receptor [14,15]. Although no TGF-α was detected in the medium from v-*sis*-transformed cells by competitive binding experiments (data not shown), we examined the effect of EGF pretreatment at 37°C on subsequent EGF binding at 4°C to high affinity receptors in both control and protein kinase C-depleted cells. The results indicate that EGF did not cause complete inhibition of EGF binding in protein kinase C-depleted cells either alone or in conjunction with PDGF (Fig. 7). Thus, neither PDGF, nor TGF-α type factors mimic the protein kinase C-independent action of the media from v-*sis*-transformed cells.

DISCUSSION

The results presented here demonstrate that growth and transforming factors can alter the properties of the EGF receptor by a number of mechanisms. A scheme depicting some of these pathways is illustrated in Figure 8. PDGF, which stimulates phosphatidylinositol turnover and generation of the second messengers diacylglycerol and calcium, acts primarily through a protein kinase C-dependent pathway. It is possible that the released calcium slightly potentiates the residual protein kinase C in PDBu-pretreated cells or has other effects. EGF, through effects on EGF binding to the high affinity receptor or internalization, acts primarily in a protein kinase C-independent manner. Finally, there are at least two components to the action of media from v-*sis*-transformed cells on EGF receptor binding. The first factor, which is stable and presumably related to p28^{sis}, is mimicked by PDGF and appears to be

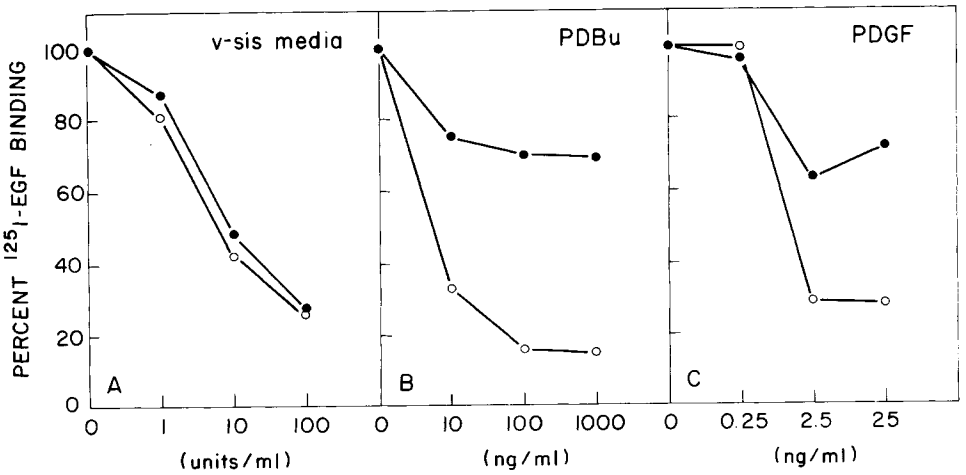

Fig. 4. Dose response of inhibition of ^{125}I-EGF binding to high affinity receptors in Swiss 3T3 cells treated with PDBu, PDGF, or medium from v-*sis*-transformed cells. Confluent quiescent Swiss 3T3 cells that had been treated for 48 hrs with 0 (○) or 100 (●) ng/ml PDBu were treated for 15 min at 37°C with various doses of **A)** medium from v-*sis*-transformed cells, **B)** PDBu or **C)** PDGF and assayed for ^{125}I-EGF binding at 4°C. The data are expressed as the percentages of ^{125}I-EGF binding in cells treated with either PDBu or medium from v-*sis*-transformed cells relative to untreated control cells. In A, the specific binding of ^{125}I-EGF constituting 100% ranged from 700 to 1,100 cpm for control and PDBu-pretreated cells; in B, representative values for the specific binding of EGF constituting 100% were 3,894 ± 184 cpm for control cells and 4,260 ± 321 cpm for PDBu-pretreated cells. Nonspecific binding was approximately 100 cpm. Each point represents the mean of triplicate samples. Standard deviations were less than 10%. The concentration of ^{125}I-EGF used in these studies was 0.05–0.1 nM.

dependent on levels of protein kinase C in the cells. The labile, second factor in the media from v-*sis*-transformed cells is required for total protein kinase C-independent changes in EGF receptor properties. The evidence suggests this is a novel factor distinct from PDGF-like peptides (p28^{sis}), EGF-like peptides (TGF-α) or TGF-β.

In interpreting these results we have considered the limitations imposed by the technique used to deplete cells of protein kinase C. The properties of the pathways involved in modifying EGF receptor properties, which are depicted in Figure 8, may be altered as a consequence of prolonged exposure to PDBu. Clearly enzymatic and immunoprecipitation assays show that levels of protein kinase C are dramatically reduced by such treatment [16,17]. However, it is likely that there are other changes that directly affect the ability of the growth factors, transforming factors, and tumor promoters to modulate EGF receptor properties. We have observed that the cellular concentration of protein kinase C required for maximum inhibition of EGF receptor binding is four to five times greater in control cells than in cells treated with tumor promoters for prolonged periods of time [25]. Similar discrepancies with respect to ^{3}H-PDBu binding and EGF receptor modification in control and C kinase-depleted cells have been reported [18]. Together, these findings indicate that secondary changes that sensitize cells to activators of protein kinase C can be induced by prolonged exposure to tumor promoters.

Despite the possibility of secondary changes, it does appear that protein kinase C is involved in the action of conditioned medium from v-*sis*-transformed cells and PDGF. In the present studies, the extent of inhibition of EGF binding in cells depleted

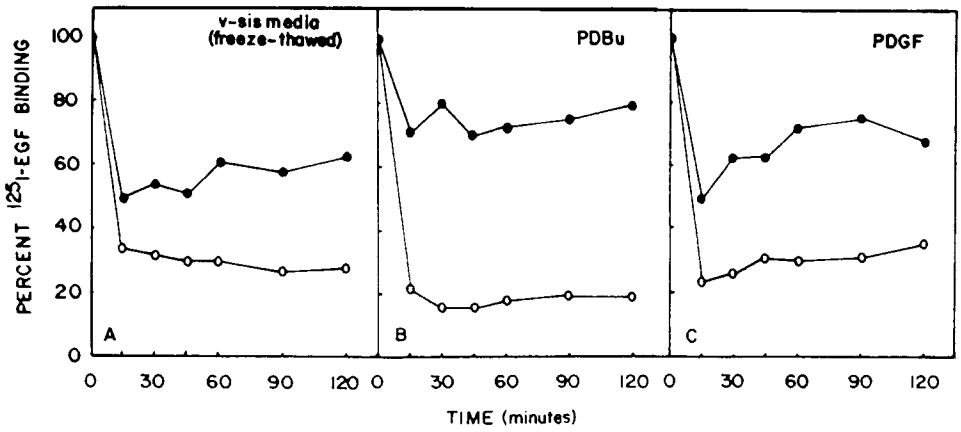

Fig. 5. Comparison between the time course of action of freeze-thawed medium from v-*sis*–transformed cells, PDBu, and PDGF in blocking high affinity ^{125}I-EGF binding to Swiss 3T3 cells. Confluent quiescent Swiss 3T3 cells that had been incubated for 48 hr with 0 (○) or 100 (●) ng/ml PDBu were treated with **A)** medium from v-*sis*–transformed cells (50 units/ml), **B)** PDBu (100 ng/ml), or **C)** PDGF (25 ng/ml) for up to 2 hr at 37°C and then assayed at 4°C for ^{125}I-EGF binding to high affinity EGF receptors. The data are expressed as the percentages of ^{125}I-EGF binding in cells treated with freeze-thawed medium from v-*sis*–transformed cells relative to untreated control cells. Representative values for the specific binding of EGF constituting 100% were 3,894 ± 184 cpm for control cells and 4,260 ± 321 cpm for PDBu-pretreated cells. Nonspecific binding was approximately 100 cpm. Each point represents the mean of triplicate samples. Standard deviations ranged from 5 to 10%. The concentration of ^{125}I-EGF used in these studies was approximately 0.1 nM.

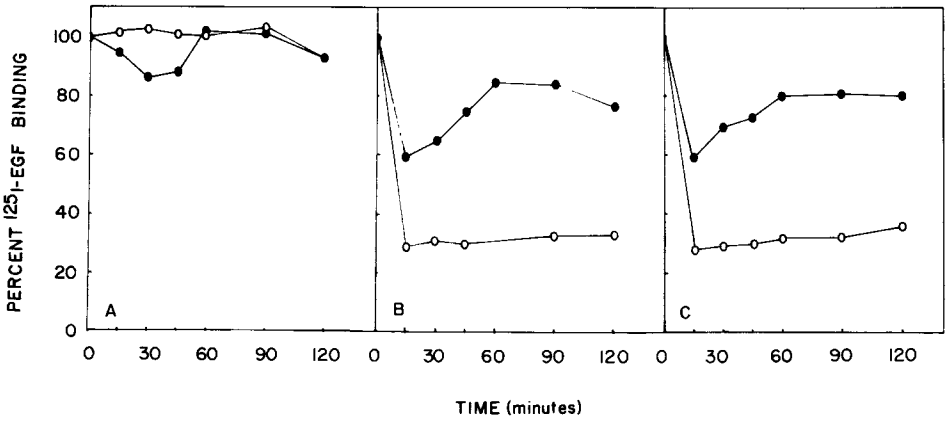

Fig. 6. Time course of specific binding of ^{125}I-EGF to high affinity receptors following treatment with TGF-β and/or PDGF. Confluent quiescent Swiss 3T3 cells that had been incubated for 48 hr with 0 (○) or 100 (●) ng/ml PDBu were treated with **A)** TGF-β (8 ng/ml), **B)** TGF-β (8 ng/ml plus PDGF) [2.5 ng/ml], or **C)** PDGF (2.5 ng/ml) alone for up to 2 hr at 37°C. Cells were then assayed at 4°C for ^{125}I-EGF binding to high affinity EGF receptors as described in Materials and Methods. Other conditions were as described in Figure 5.

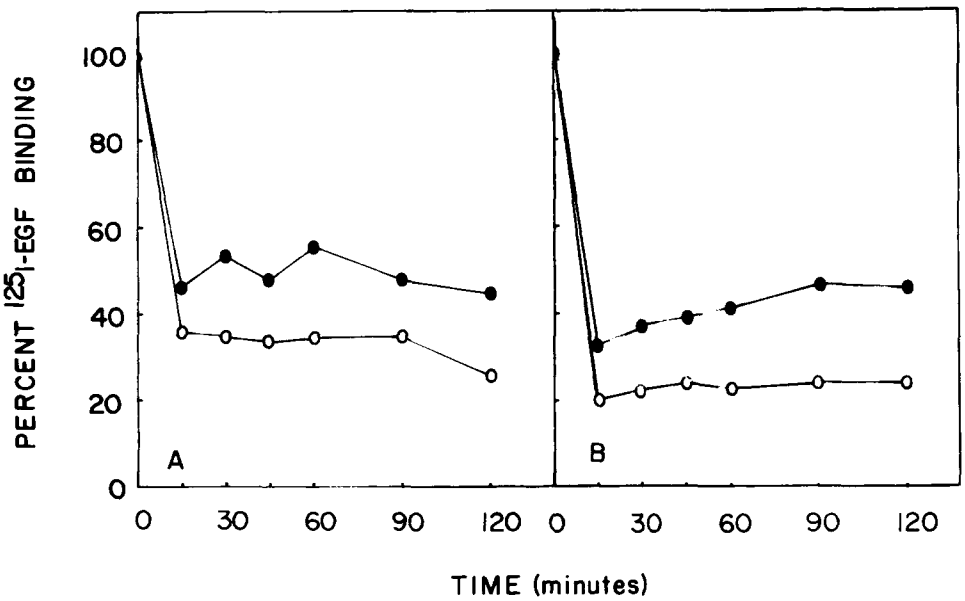

Fig. 7. Time course of specific binding of ^{125}I-EGF to high affinity receptors following treatment of EGF alone or in combination with PDGF. Confluent quiescent Swiss 3T3 cells that had been incubated for 48 hr with 0 (○) or 100 (●) ng/ml PDBu were treated with **A)** EGF (1 ng/ml) or **B)** EGF (1 ng/ml) plus PDGF (2.5 ng/ml) for up to 2 hr at 37°C. Cells were then assayed at 4°C for ^{125}I-EGF binding to high affinity EGF receptors. Other conditions were as described in Figure 5.

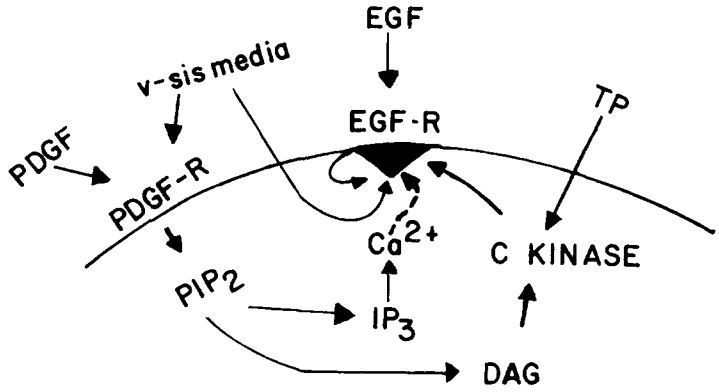

Fig. 8. Scheme depicting multiple pathways by which growth factors may modify the EGF receptor. Tumor promoters (TP) modulate EGF receptor properties through direct activation of protein kinase C. Presumably, the stable, PDGF-like factors in media from v-*sis*-transformed cells (v-*sis* media) bind to the PDGF receptor (PDGF-R), and by so doing stimulate phosphatidylinositol biphosphate (PIP_2) breakdown to diacylglycerol (DAG) and inositol triphosphate (IP_3). In addition to PDGF-like peptides, untreated media from v-*sis*-transformed cells contains a labile factor required for modification of EGF receptor properties in a protein kinase C-independent manner. The role of calcium in this pathway has not been elucidated.

of protein kinase C is less than that observed in control cells. Thus, these cells appear to be desensitized to the action of PDGF and freeze-thawed medium from v-*sis*–transformed cells on EGF receptor binding. PDGF and very likely the PDGF-like peptides in the medium from v-*sis*–transformed cells generate the endogenous activator of protein kinase C, diacylglycerol [19]. Therefore, the loss of potency of these agents in protein kinase C-depleted cells is consistent with the involvement of protein kinase C in their mechanism of action.

The role of protein kinase C in the action of EGF is not understood. In A431 cells, limited phosphatidylinositol turnover has been reported [20], leading us to postulate that protein kinase C may act in a limited manner as a feedback inhibitor of the EGF receptor [1]. Whether significant phosphatidylinositol turnover occurs in Swiss 3T3 cells is less certain. Habenicht et al [19] found a small but discrete level of diacylglycerol in this cell type following EGF treatment. However, recent evidence from a number of investigators suggests there is no significant phosphatidylinositol turnover in Swiss 3T3 cells treated with EGF [21,22]. The data we have obtained point to a limited role for protein kinase C, or some enzyme sensitive to PDBu down-modulation, in the action of EGF on its own receptor. Since, in Swiss 3T3 cells, EGF can trigger an increase in cellular calcium levels derived from extracellular sources [22], it is possible that protein kinase C is being activated by EGF in this cell type to a limited extent through a calcium-related mechanism. However, it is likely that there are other pathways that constitute the major route for desensitization or feedback inhibition of the EGF receptor by EGF.

The particular signal-transducing pathways that are activated by growth factors such as PDGF and TGF-β may be cell-type specific. For example, in Balb/C 3T3 cells PDGF inhibits EGF binding in the complete absence of protein kinase C [26], whereas in Swiss 3T3 cells we note a dependence on this kinase. Similarly, in other cell types, TGF-β causes a rapid inhibition of high affinity EGF binding [12,13] that is not observed in Swiss 3T3 cells (see Results). This lack of responsiveness occurs with concentrations of TGF-β known to bind specifically to Swiss 3T3 cells [23]. Finally, we do not observe the synergism between TGF-β and PDGF that has been reported for other cell types [24]. Thus, there are clearly multiple mechanisms for achieving similar endpoints, and not all these pathways are operational in every cell type.

ACKNOWLEDGMENTS

We thank J. Porter and C. Stiles for providing the media from NRK-transformed cells, R. Assoian for the gift of purified PDGF, S. Decker for the antiEGF receptor antisera, P. McCaffrey for helpful comments, and E. Fahci for assistance in preparing the manuscript. This work was supported by National Cancer Institute Awards CA35541 and CA40407 to M.R.R.

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