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Heterologous Regulation of EGF Receptors in Fibroblastic Cells

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Platelet-derived growth factor (PDGF) increases the mitogenic activity of epidermal growth factor (EGF) in several cells lines, including BALB/C-3T3. PDGF-treated BALB/C-3T3 cells manifest a reduced capacity to bind ^{125}I-labeled EGF due to a loss of high affinity EGF receptors. Cholera toxin potentiates the ability of PDGF to both decrease EGF binding and initiate mitogenesis. Whether PDGF increases EGF sensitivity via its effects on EGF receptors is not known and requires a more complete understanding of the mechanism by which PDGF decreases EGF binding.

12-O-tetradecanoylphorbol 13-acetate (TPA) also reduces EGF binding in BALB/C-3T3 and other cells, presumably by activating protein kinase C and, consequently, inducing the phosphorylation of EGF receptors at threonine−654. PDGF indirectly activates protein kinase C, and EGF receptors in PDGF-treated WI-38 cells are phosphorylated at threonine-654. Thus, the effects of PDGF on EGF binding may also be mediated by protein kinase C. We investigated this hypothesis by comparing the actions of PDGF and TPA on EGF binding in density-arrested BALB/C-3T3 cells.

Both PDGF and TPA caused a rapid, transient, cycloheximide-independent loss of ^{125}I-EGF binding capacity. The actions of both agents were potentiated by cholera toxin. However, whereas TPA allowed EGF binding to recover, PDGF induced a secondary and cycloheximide-dependent loss of binding capacity. Most importantly, PDGF effectively reduced binding in cells refractory to TPA and devoid of detectable protein kinase C activity. These findings indicate that PDGF decreases EGF binding by a mechanism that involves protein synthesis and is distinct from that of TPA.

Key words: epidermal growth factor, platelet-derived growth factor, tumor promoters, growth stimulation, growth factor receptors, cyclic AMP

The ability of nontransformed cells in culture to proliferate in response to a given mitogen is often dependent upon prior or concomitant sensitization by a second mitogen. Density-arrested BALB/C-3T3 cells, for example, do not respond proliferatively to epidermal growth factor (EGF) unlesss pretreated or co-incubated with

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platelet-derived growth factor (PDGF). In this system, PDGF initiates proliferation by rendering quiescent cells "competent" to re-enter the cell cycle [1]. EGF in the presence of somatomedin C (SmC) mediates the subsequent G_0/G_1 traverse of competent cells [2]. EGF and SmC also promote the progression of quiescent NRK, AKR, 10T½ and Swiss 3T3 cells; PDGF, while not obligatory for the traverse of these cells, reduces the concentration of EGF required for their stimulation [3–5]. The mechanism by which PDGF sensitizes cells to EGF is not known.

Competence formation requires only a brief exposure (2–4 hr) of cells to PDGF. Once rendered competent, cells remain responsive to EGF and SmC for several hours following the removal of PDGF from the culture medium [1,6]. This observation implies the existence of stable PDGF-induced changes in the intracellular environment that denote the competent state. The addition of PDGF to target cells elicits a variety of responses including altered distribution of cytoskeletal components such as vinculin [7,8], preferential gene transcription and protein synthesis [9–13], and changes in ion transport [14–15], and phospholipid metabolism [16–19]. Although each of these events represents a potential competence signal, or may be involved in the generation of that signal, a relationship between these processes and the subsequent actions of EGF has yet to be defined.

HETEROLOGOUS RECEPTOR MODULATION

PDGF and EGF communicate with target cells via specific receptors located in the plasma membrane; hormone-bound receptors are rapidly internalized and ultimately degraded [20,21]. Cellular capacity to bind ^{125}I-EGF is reduced following treatment with PDGF, the result of an indirect action of PDGF on either the number or affinity of EGF receptors [22–27]. This finding is paradoxical, given the synergistic effects of PDGF and EGF on mitogenesis and contrasts with data showing increased SmC binding in PDGF-treated BALB/C-3T3 cells [28]. The ability of PDGF to modulate EGF binding demonstrates, however, that EGF receptors are targets of both PDGF and EGF and, thus, potential mediators of the effects of PDGF on EGF sensitivity.

In support of this hypothesis, Wharton et al [23] and Leof et al [29] have shown that cholera toxin and other agents that increase cellular levels of cyclic AMP enhance both PDGF-induced competence formation and the loss of EGF binding capacity produced by PDGF. Treatment of density-arrested BALB/C-3T3 cells with optimal levels of PDGF produced, at most, a 60–70% loss of EGF binding capacity, whereas PDGF in combination with cholera toxin reduced ^{125}I-EGF binding greater than 90%. Moreover, cells treated with both PDGF and cholera toxin no longer required EGF for G_0/G_1 traverse [23].

In the absence of PDGF, cyclic AMP elevating agents also act synergistically with EGF to promote the G_0/G_1 traverse of BALB/C-3T3 cells and other cell lines [4,30–33]. In addition, comparison of three nontransformed cell lines revealed a direct correlation between cyclic AMP content at quiescence and ability to respond mitogenically to EGF [4]. In contrast to PDGF, however, agents increasing cellular cyclic AMP levels did not affect the binding of EGF to its receptors; down regulation and degradation of EGF-receptor complexes were also unaffected by these agents [4,34]. Thus, the mechanism by which cyclic AMP increases EGF sensitivity may differ from that of PDGF.

COMPARISON OF THE EFFECTS OF PDGF AND TPA ON EGF BINDING

To address the question of whether PDGF potentiates EGF sensitivity by modulating EGF receptors, experiments were performed to determine the mechanism by which PDGF decreases EGF binding. Like PDGF, the potent tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA) also reduces EGF binding in several cell lines [35–48] and initiates the proliferation of density-arrested BALB/C-3T3 cells [49]. When added to intact cells, both agents induce the phosphorylation of EGF receptors at threonine-654 [50–51]. Threonine-654 is also phosphorylated in vitro by protein kinase C [52], the binding site and cellular mediator of TPA [53]. PDGF indirectly stimulates this kinase by increasing the turnover of phosphatidylinositol and the formation of diacylglycerol, the endogenous activator of protein kinase C [16–19]. Phosphorylation of purified EGF receptors by protein kinase C has been shown to reduce the affinity of these receptors for EGF [54]. These observations suggest that PDGF and TPA decrease EGF binding by a common process involving the activation of protein kinase C and the consequent phosphorylation of EGF receptors at threonine-654. Direct confirmation that TPA reduces EGF binding by this mechanism is provided by experiments showing that cells transfected with EGF receptor cDNA containing Ala-654 did not exhibit a loss of EGF binding capacity in response to TPA [55].

To determine whether PDGF acts like TPA, we compared the actions of PDGF and TPA on EGF binding in BALB/C-3T3 cells. In the first set of experiments, density-arrested cells were incubated at 37°C in medium containing 25 ng/ml PDGF or 500 nM TPA. At various times, the monolayers were rinsed and ^{125}I-EGF binding was measured at 4°C. In response to PDGF, ^{125}I-EGF binding decreased to within 30% of control (quiescent, nonrefed cultures) within 15 min, increased to 50% by 45–60 min, and between 1 and 3 hr, declined from 50% to 25% (Fig. 1). Binding capacity remained at this reduced level for at least 4 hr (data not shown). TPA also rapidly and transiently reduced EGF binding; the response elicited by TPA during the first hour of treatment was greater than, but qualitatively similar to, that observed with PDGF. In contrast to PDGF, however, ^{125}I-EGF binding in the TPA-treated cells did not decline after 60 min but continued to slowly increase. PDGF, therefore, differs from TPA in its ability to effect a secondary, sustained decrease in EGF binding activity.

Previous studies have shown that prolonged exposure of cells to high levels of TPA renders cells refractory to TPA [56–59]. Cellular desensitization to TPA is accompanied by a dramatic decline in the number of binding sites for TPA [56,60]. In accord with the concept that protein kinase C is the receptor for TPA, the activity of this enzyme in TPA-pretreated cells is reduced or absent [61]. The loss of protein kinase C activity results from degradation, as opposed to inactivation, of the kinase [62]. To determine whether PDGF decreases EGF binding in the absence of protein kinase C, quiescent BALB/C-3T3 cultures were exposed to 600 nM TPA for 20 hr at 37°C under conditions nonpermissive for cell cycle traverse. The duration of the exposure was sufficient to allow EGF binding to recover to essentially control levels (Fig. 2). Such treatment resulted in the loss of detectable levels of protein kinase C activity and the consequent inability of cells to respond to TPA in terms of 1) decreasing ^{125}I-EGF binding and 2) initiating DNA synthesis (Fig. 2 and data not shown). PDGF, however, reduced EGF binding to a similar extent and in a compa-

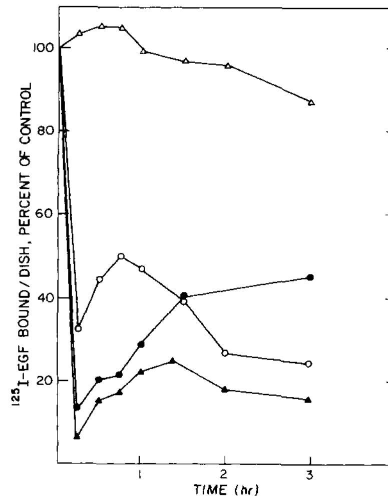

Fig. 1. Effect of PDGF or TPA on EGF binding. Density-arrested BALB/C-3T3 cells were incubated at 37°C in medium alone (△) or medium containing 25 ng/ml PDGF (○), 500 nM TPA (●), or both (▲). At various times after refeeding, the cells were rinsed with binding medium and ^{125}I-EGF binding was determined at 4°C. The amount of ^{125}I-EGF specifically bound by quiescent, nonrefed cultures represents 100%. Reproduced from Proc. Natl. Acad. Sci. USA 83:3834–3838, 1986, with permission.

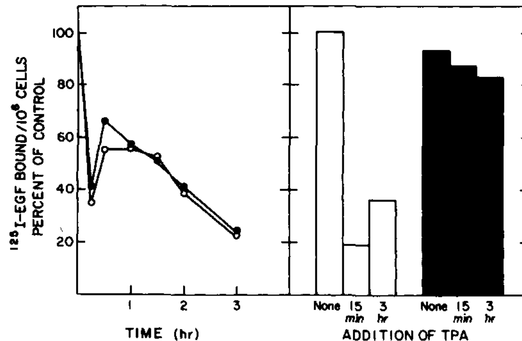

Fig. 2. Effect of PDGF or TPA on control or TPA-pretreated cells. Quiescent cultures were incubated for 20 hr in medium supplemented with 0.25% platelet-poor plasma and 600 nM TPA. TPA-pretreated (●) or nontreated control (○) cells were refed with medium containing 25 ng/ml PDGF (left panel) or 600 nM TPA (right panel). ^{125}I-EGF binding was determined at the indicated times. Reproduced from Proc. Natl. Acad. Sci. USA 83:3834–3838, 1986, with permission.

rable manner in both control and TPA-pretreated cultures. In agreement with previous reports [59–63], cells desensitized to TPA also remained mitogenically responsive to PDGF (data not shown). The ability of PDGF to reduce EGF binding in cells refractory to TPA implies that the mechanism by which PDGF modulates EGF binding differs from that of TPA and thus, unlike that of TPA, is independent of protein kinase C. Whether PDGF activates other kinases that phosphorylate EGF receptors, and whether such phosphorylation is responsible for the loss of EGF binding capacity, are at present unknown.

REQUIREMENT FOR PROTEIN SYNTHESIS AND EFFECT OF CHOLERA TOXIN

As described above, the secondary decrease in EGF binding induced by PDGF is not observed in cells receiving TPA. Unlike the initial reduction produced by TPA (or EGF) [64], this secondary loss of EGF binding capacity was strictly dependent on continued protein synthesis (Fig. 3, and data not shown). When added to cells in combination with PDGF, cycloheximide not only prevented this delayed decrease but allowed binding capacity to recover to essentially control levels. Cycloheximide had a similar effect on cells treated with both PDGF and cholera toxin, and 5,6-dichloro-β-ribofuranosylbenzimidazole, an inhibitor of mRNA synthesis, also reversed the loss of binding capacity produced by PDGF (data not shown). These observations suggest that PDGF may decrease EGF binding by two distinct processes that differ in their requirement for protein and RNA synthesis.

Although PDGF and TPA affect EGF binding dissimilarly and perhaps act via different mechanisms, the inhibitory actions of TPA on EGF binding, like those of PDGF, were enhanced by cholera toxin. When suboptimal amounts of TPA (5–50 nM) were added to cells in combination with cholera toxin, ^{125}I-EGF binding decreased at a faster rate and to a greater extent than was observed in cells receiving TPA alone (Fig. 4). Higher concentrations of TPA (500 nM) elicited a rapid and maximum response and, thus, obscured these effects of the toxin. At this concentration of TPA, however, an additional effect of cholera toxin was evident; this agent antagonized the recovery of binding that began at 1 hr in the TPA-treated cells. Regardless of the dose of TPA or of the presence of cholera toxin, ^{125}I-EGF binding increased after 3 hr; binding activity, however, remained lower in the toxin-treated cells (data not shown).

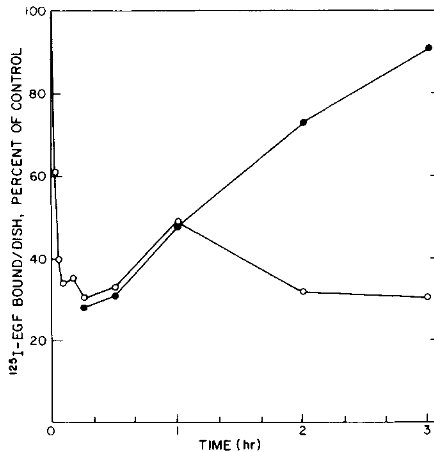

Fig. 3. Recovery of EGF binding in the presence of cycloheximide. Cycloheximide was added to cells in spent medium to a final concentration of 10 μg/ml. Fifteen minutes later, the cultures were refed with medium containing 25 ng/ml PDGF and 10 μg/ml cycloheximide (●). Cells not pre-exposed to cycloheximide received PDGF alone (○). ^{125}I-EGF binding was determined at various times after refeeding. Cycloheximide at the concentration used inhibited protein synthesis greater than 95%. Reproduced from Proc. Natl. Acad. Sci. USA 83:3843–3838, 1986, with permission.

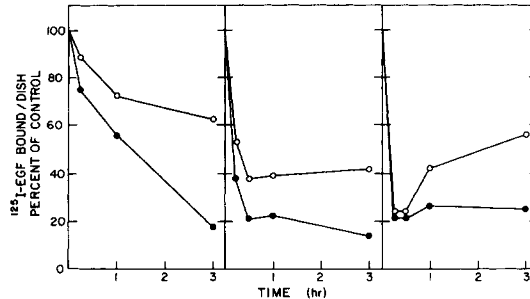

Fig. 4. Enhancement by cholera toxin of the TPA effect. Confluent cells were pretreated with medium containing 1 μg/ml cholera toxin for 2 hr. Nontreated (○) and toxin-treated cells (●) were then refed with medium supplemented with 5 nM (left panel), 50 nM (middle panel) or 500 nM (right panel) TPA; cells pre-exposed to cholera toxin also received the toxin at 1 μg/ml. ^{125}I-EGF binding activity was measured at various intervals after refeeding. Reproduced from Proc. Natl. Acad. Sci. USA 83:3834–3838, 1986, with permission.

CONCLUDING REMARKS

The data presented here and in previous reports [23,29] demonstrate that the effects of PDGF on EGF binding in density-arrested BALB/C-3T3 cells are 1) potentiated by agents that increase cellular levels of cyclic AMP, 2) are dependent, in part, on protein synthesis, and 3) presumably do not require protein kinase C. In addition, the data show that PDGF-induced competence formation and changes in EGF binding are correlated in two respects: 1) cholera toxin potentiated the actions of PDGF on both processes and 2) TPA pretreatment did not abrogate the effects of PDGF on either process. In contrast, as reported by Collins and Rozengurt [59], vasopressin decreased EGF binding and stimulated DNA synthesis in quiescent, but not in TPA-pretreated, Swiss 3T3 cells. A definitive relationship between heterologous receptor modulation and mitogenesis, however, has yet to be demonstrated and requires a more complete understanding of the mechanism by which PDGF decreases EGF binding and knowledge of the fate of EGF receptors in PDGF-treated cells.

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