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CYCLIC AMP POTENTIATES DOWN REGULATION OF EPIDERMAL GROWTH FACTOR RECEPTORS BY PLATELET-DERIVED GROWTH FACTOR

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Summary: Pretreatment of Balb/c-3T3 cells with platelet-derived growth factor (PDGF) has been shown to decrease binding sites for ^{125}I-labelled epidermal growth factor (EGF) (1,2,3). Agents which elevate cellular cyclic AMP concentrations enhance this ability, and the change in EGF binding is inversely proportional to the elevation of cyclic AMP. In quiescent density arrested cells, the sensitivity of cells to down regulation of EGF receptors by PDGF is proportional to the cyclic AMP content of the cultures in three different cell lines. Agents which elevate cyclic AMP and which potentiate PDGF mediated heterologous down regulation of EGF receptors are able, like cholera toxin (3), to stimulate cells to synthesize DNA in defined medium in the absence of EGF. Down regulation of EGF receptors by PDGF in combination with agents elevating cyclic AMP effectively mimics the action of EGF.

PDGF, EGF, and somatomedin-C (or high concentrations of insulin) are potent growth factors for fibroblasts, but critical biochemical actions of each and their unique actions in promoting growth are under study. These three factors in combination will support G_1 traverse and DNA synthesis in defined medium (4) and can be used to study control of cell cycle traverse. The mechanisms by which these agents act in concert to produce the mitogenic response are unknown. PDGF is known to act prior to progression (5) and its persistent action can be demonstrated after transient exposure to cells (6). In contrast, EGF is required during progression during the first 6 hours of G_1 traverse in

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density-arrested Balb/c-3T3 cells and must be continuously present (4,7,8). It has been shown that cholera toxin (choleragen) can enhance the mitogenic response in Swiss 3T3 cells (9,10), mammary epithelial cells (11), and keratinocytes (12,13). Wharton et al. (14) found that in Balb/c-3T3 cells choleragen stimulated G_1 traverse and DNA synthesis by potentiating PDGF action during competence formation but had no effect during progression. After Wrann et al. found that platelet factor could down regulate EGF receptors (1), which was confirmed by Heldin et al. (2), we found that choleragen enhanced this heterologous down regulation of EGF receptors by PDGF (3). Since PDGF with choleragen, like EGF, resulted in loss of EGF receptors from the cell surface, we speculated that the action of PDGF with choleragen might effectively substitute for the requirement for EGF in progression to S phase of the cell cycle. It was confirmed that in cells so treated, EGF was no longer required, and cells synthesized DNA in defined medium with only insulin added (3). In the present study we show that several agents elevating cyclic AMP, like choleragen, potentiate both the ability of PDGF to down regulate EGF receptors and the ability of cells to synthesize DNA in defined medium in the absence of EGF. The ability to elevate cyclic AMP concentrations is proportional to the degree of down regulation of EGF receptors. Down regulation of the EGF receptor by platelet factor in combination with agents elevating cyclic AMP, perhaps by effecting the biochemical actions of EGF, abrogates the requirement for EGF and suggests that events leading to down regulation or down regulation, and possibly internalization, of receptors may be necessary events for the mitogenic action of EGF.

MATERIALS AND METHODS

Materials. EGF was purified from submaxillary glands of adult male mice according to Savage and Cohen (15) and iodinated with ^{125}I[NaI] (Amersham) (16) to a specific activity of 2.0 Ci/umole using Chloramine T. Choleragen and choleragenoid were from Calbiochem-Behring, and 8-bromo cyclic AMP, PGF_2 alpha and isobutylmethylxanthine were from Sigma. [Methyl-^{3}H] thymidine (6.7 Ci/umole) was from ICN. Tissue culture media and materials were from Flow Laboratories (McLean, Virginia). Antibody to cyclic AMP was from Biotech, Inc. (Shawnee Mission, Kansas).

Cells and Cell Culture. AKR and NRK cells were obtained from Dr. H. Moses. All cells were grown in Dulbecco-Vogt's modified Eagle's medium (DME) with 10% calf serum (Colorado Serum Company, Denver, Colorado), 50 units penicillin

per ml, 50 ug streptomycin per ml, and 4 mM glutamine in a humidifed atmosphere of 5% CO_2 - 95% air at 37°C.

EGF Binding Assays (3). Cells were plated at 5 x 10^4 cells per 35 mm dish and fed 3 days later. Cells were allowed to reach confluence and then used 4-6 days later for experiments. Medium was changed to DME containing additions as shown and 0.25% platelet poor plasma (5,6) and incubated for 3 hours at 37°C. Medium was changed to 0.6 ml binding medium (Hanks' balanced salt solution with 20 mM HEPES and 0.1% bovine serum albumin) at 4°C. [^{125}I] EGF was added (4 x 105 cpm, 4.4 x 10^{-10}M) to triplicate dishes for 4 hours at 4°C. Nonspecific binding was determined in the presence of 1 ug/ml EGF. The assay was termined by washing the cells 3 times with 2 ml binding medium, solubilizing cells with 1% SDS/0.1 N NaOH and counting radioactivity in a gamma counter.

DNA Synthesis Assays (14). Cells were subcultured in Nunc (0.3 cm^2) 96 well microtiter plates in 0.2 ml DME with 10% calf serum until confluent. Three days later DME containing 5 uCi/ml [methyl-^{3}H]thymidine and appropriate supplements were added. DNA synthesis was stopped after 30 hours by the addition of 60 ul 1 M ascorbic acid. Plates were washed with phosphate buffered saline and fixed with methanol twice for 10 minutes. After being washed with water cells were processed for autoradiography according to Antoniades et al. (17).

Cyclic AMP Assays. Confluent cultures in 35 mm dishes were exposed to additions as noted in tables and washed at 4°C with DME. Cells were treated with 5% trichloroacetic acid for 16 hours at 4°C, and extracted cyclic AMP was purified on Dowex 50 X-8 resin according to Krishna (18). Cyclic AMP was measured by the method of Steiner et al. (19).

RESULTS

In order to determine whether different agents known to elevate cyclic AMP could enhance PDGF-mediated down regulation of EGF receptors, density-arrested Balb/c-3T3 cells were incubated with the agents shown in Table I in combination with PDGF for 3 hours prior to binding assay. The data show the percent increase in down regulation produced by these agents in comparison with that produced by PDGF alone. Choleragenoid, the binding subunit of choleragen, which failed to elevate cyclic AMP (data not shown) did not alter EGF binding when added together with PDGF. Prostaglandin F_2 alpha, 8-bromo cyclic AMP, isobutylmethylxanthine (IBMX), and choleragen, which elevate intracellula cyclic AMP by different mechanisms, were all able to potentiate the action of PDGF. In control experiments without PDGF, these agents did not alter EGF binding.

Though all the agents listed in Table I except choleragenoid are known to elevate cyclic AMP concentrations, the data do not disprove the possibility that they might act through other mechanisms. To substantiate that the

Table I
POTENTIATION OF PDGF-INDUCED DOWN REGULATION
OF EGF RECEPTORS BY AGENTS THAT INCREASE
CELLULAR CYCLIC AMP LEVELS

Treatment (Plus PDGF)	% Increase
CHOLERAGENOID (1 ug/ml)	3
8-BROMO-CYCLIC AMP (1 mM)	15
PROSTAGLANDIN F_2 ALPHA (1 uM)	56
ISOBUTYLMETHYLXANTHINE (0.1 mM)	66
CHOLERAGEN (1 ug/ml)	109

Quiescent cultures of Balb/c-3T3 cells were incubated for 3.5 h at 37°C in DME containing 0.25% platelet-poor plasma with the indicated additions plus 20 ug/ml PDGF. Cultures were then washed and incubated at 4°C for the ^{125}I-EGF binding assay as in Materials and Methods. The data show the percent increase in PDGF potentiated down regulation of EGF receptors mediated by the additions compared to that by PDGF alone. PDGF alone decreased EGF binding by an average of 31% in 18 determinations.

potentiation of PDGF-induced down regulation was mediated by cyclic AMP, the percent decrease in EGF binding compared to untreated controls was determined after treatment with PDGF or PDGF in combination with different concentrations of choleragen and IBMX. Duplicate cultures treated in parallel were extracted with 5% trichloracetic acid and cyclic AMP content of the cultures determined by radioimmunoassay (19). As shown in Table II,

Table II
INCREASES IN CYCLIC AMP
POTENTIATE THE PDGF INDUCED DOWN
REGULATION OF EGF RECEPTORS

Fold Increase in Cyclic AMP	Percent Decrease in EGF Binding
1 (PDGF Alone)	29.67 ± 3.27
1 - 3	35.20 ± 4.79
4 - 9	63.05 ± 3.31
10 - 14	65.50 ± 2.56

Quiescent cultures of density arrested Balb/c-3T3 cells were treated with DME containing 0.25% platelet poor plasma and 15 ug/ml PDGF. Other plates received in addition 0.03 - 3.0 ug choleragen per ml and 0.01-0.10 mM IBMX. After 3.5 h at 37°C the cells were assayed for ^{125}I-EGF binding as in Materials and Methods. Parallel cultures were treated with identical concentrations of PDGF, choleragen, and IBMX for 3.5 h at 37°C. The plates were washed with Hanks' balanced salt solution and cyclic AMP extracted, purified, and assayed as in Materials and Methods. EGF binding was not altered in samples with elevated cyclic AMP (1-14 fold) if PDGF was not present. Results show mean ± S.E.M.

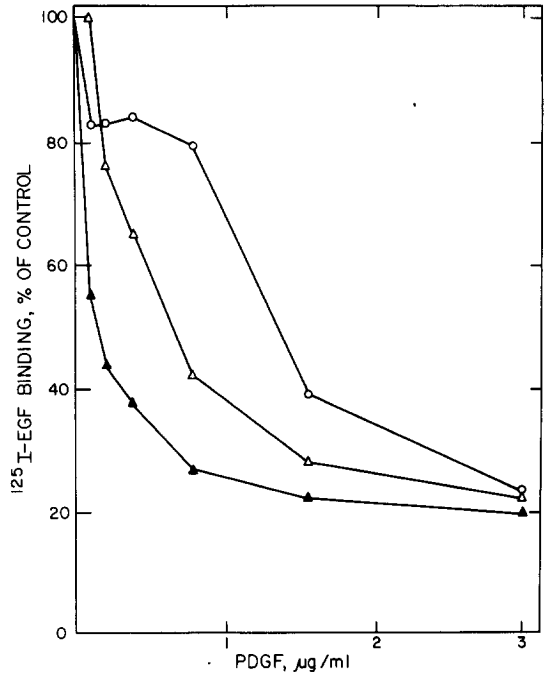

Figure 1. Cells were incubated with PDGF as indicated in DME containing 0.25% plasma for 3.5 h at 37°C and assayed for ^{125}I-EGF binding as in Materials and Methods. Control cells bound 7.8 (Balb/c-3T3), 12.0 (AKR), or 10.9 (NRK) femtomoles ^{125}I-EGF per 10^6 cells. Balb/c-3T3, - O - ; ARK, - Δ -; NRK, - ▲ -.

as the cyclic AMP content increased up to nine-fold over treatment with PDGF alone, there was a corresponding increase in the ability of PDGF to decrease EGF binding.

To determine whether differences in cyclic AMP content might affect the response to PDGF in untreated cultures, three fibroblast lines with different cyclic AMP contents at density arrest were examined for their ability to down regulate EGF receptors after treatment with PDGF. These lines when cultured under identical conditions demonstrated proportionality of cyclic AMP contents of unstimulated cultures and the sensitivity to PDGF-induced down regulation of EGF binding. Thus cyclic AMP content was greatest for NRK cells (23.9 pmoles/ 10^6 cells), which were most sensitive to the ability of PDGF to down regulate EGF receptors, and lowest in Balb/c-3T3 cells (8.4 pmoles/10^6 cells) which were least sensitive. AKR cells were intermediate in both assays (10.2 pmoles/ 10^6 cells). AKR cells required three times, and Balb/c-3T3 cells over 6 times, the PDGF concentration needed by NRK cells for 50% down regulation of EGF

Table III
ABROGATION OF THE REQUIREMENT FOR EGF
FOR DNA SYNTHESIS IN BALB/C-3T3 CELLS IN DEFINED MEDIUM

Addition to PDGF and Insulin	Percent Labelled Nuclei
NONE	6 ± 2
8-BROMO-CYCLIC AMP (1 MM)	27 ± 3
PGF_2 ALPHA (1 mM)	37 ± 2
IBMX (0.1 mM)	40 ± 2
CHOLERAGEN (1 ug/ml)	73 ± 4

Cells grown in 96-well trays were cultured with additions as noted and [methyl-^{3}H]thymidine for DNA synthesis assay as in Materials and Methods. Cells treated similarly with PDGF and 5% plasma were found to have 72 ± 4% labelled nuclei in 10 determinations. Results show mean ± S.E.M.

receptors. This differential sensitivity was not apparent in the proportion of receptors down regulated by PDGF, which was similar in all three lines at high PDGF concentrations, but required submaximal PDGF concentrations for its demonstration (Fig. 1).

In previous studies a defined medium composed of DME, EGF (2×10^{-9}M), and insulin (10^{-5}M) was shown to be able to support progression to S phase in Balb/c-3T3 cells after treatment with PDGF (4). In cells treated with both PDGF and choleragen, however, which have minimal residual EGF binding (3), EGF was no longer required for DNA synthesis (3). We therefore examined the ability of agents elevating cyclic AMP concentrations to sustain progression through G_1 in Balb/c-3T3 cells exposed to PDGF and insulin (10^{-5}M). As shown in Table III, 8-bromo cyclic AMP, prostaglandin F_2 alpha, and IBMX enhanced DNA synthesis over controls, although choleragen was most effective and was as effective as PDGF and plasma, in stimulating DNA synthesis. The ability of agents elevating cyclic AMP by different mechanisms to act, although less effectively, like choleragen suggests that choleragen is acting through stimulation of adenylate cyclase.

DISCUSSION

These data confirm and extend the finding that choleragen enhances PDGF mediated down regulation of EGF receptors (3). An analog of cyclic AMP, a prosta-

glandin capable of stimulating adenylate cyclase, and a phosphodiesterase inhibitor were able to enhance PDGF-induced down regulation of EGF receptors but were inactive without PDGF, indicating that choleragen was acting through cyclic AMP rather than through an unknown or nonspecific effect on the cell membrane. Furthermore, the increase in cyclic AMP produced by these agents was proportional to the enhancement of down regulation. Agents elevating cyclic AMP were without effect on EGF binding alone and were only effective by virtue of their ability to enhance the action of PDGF.

It has been demonstrated that the ability of choleragen to stimulate DNA synthesis is mediated by PDGF and is related to the state of competence. Choleragen added prior to progression is effective and potentiates the action of PDGF, but choleragen added after removal of PDGF or during progression is ineffective (14). Density arrested Balb/c-3T3 cells pre-treated with PDGF or PDGF and choleragen do not synthesize DNA earlier than 12 hours after addition of plasma and therefore can be said not to progress toward S phase. This action of choleragen was shown to be mimicked by agents elevating cyclic AMP (14). The ability of choleragen (a) to enhance DNA synthesis by potentiating the action of PDGF and (b) to enhance PDGF-mediated down regulation of EGF binding may indicate that down regulation of EGF receptors, or events preceding their down regulation, are required for stimulation of DNA synthesis.

Since the oligomeric binding subunit of choleragen binds to gangliosides and can cluster in the plane of the membrane (20), it is possible that nonspecific perturbations unrelated to adenylate cyclase stimulation could affect PDGF-mediated down regulation of EGF receptors. This possibility is excluded by the failure of choleragenoid to enhance the action of PDGF on EGF binding (Table I) and by the demonstration that agents elevating cyclic AMP by diverse mechanisms are able to mimic the action of choleragen. Furthermore, these agents substitute for choleragen, although they are less effective, in effects on both EGF binding (Table II) and DNA synthesis (Table III).

The relevance of the cyclic AMP content of cells treated with PDGF is suggested by the differential sensitivity of Balb/c-3T3, AKR, and NRK cells to PDGF as assessed by binding of ^{125}I-EGF. Cells with greater resting cyclic AMP contents showed more effective down regulation of EGF receptors by subsaturating concentrations of PDGF. Not only cells whose cyclic AMP has been elevated by treatment but also those with naturally greater cyclic AMP contents are more sensitive to down regulation of the EGF receptor by PDGF. The importance of this differential sensitivity is extended by the finding that NRK, AKR and Balb/c-3T3 cells show differential sensitivity to the mitogenic action of PDGF in proportion to the resting cyclic AMP contents (data not shown). Wharton et al. showed that sensitivity to mitogenic action of PDGF was potentiated by experimentally induced increases in cyclic AMP (14).

Finally, the data show that density-arrested Balb/c-3T3 cells treated with PDGF and agents elevating cyclic AMP have a decreased or absent requirement for EGF. There are data to suggest that EGF may not be required to effect the action of the natural ligand. First, antibody to the receptors for insulin (21) and for EGF (22) can mimic certain actions of the peptides. Second, cyanogen-bromide cleaved EGF, which is inactive as a mitogen, is mitogenic after cross-linking by anti-EGF antibody (23). Thus, appropriate modulation of the receptor may mimic EGF action. The EGF receptor may be modulated in the absence of EGF, and this modulation may effectively substitute for EGF. Similar events may occur in EGF receptor down regulation in liver regeneration (24) or after viral transformation (25). The possibility is suggested by our data that the action of PDGF with agents elevating cyclic AMP may, in down regulating the EGF receptor, result in changes which would be produced by EGF itself, thereby substituting for EGF.

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