Coupling of Epidermal Growth Factor (EGF) with the Antiproliferative Activity of cAMP Induces Neuronal Differentiation*

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Nerve growth factor (NGF) functions as a progression factor with both mitogenic and antimitogenic activities. When PC12 cells are treated with NGF, they advance to the G1 stage of the cell cycle before they differentiate. The correlation between cessation of proliferation and differentiation suggests that the antimitotic activity of NGF may be obligatory for differentiation. Although epidermal growth factor- (EGF) and NGF-treated PC12 cells share several common properties, including activation of the mitogen-activated protein (MAP) kinase pathway and induction of immediate early genes, EGF is mitogenic for PC12 cells and does not normally stimulate differentiation. However, combinations of EGF and low levels of cAMP stimulate differentiation even though neither agent alone does (Mark, M. D., Liu, Y., Wong, S. T., Hinds, T. R., and Storm, D.R. (1995) J. Cell Biol. 130, 701–710). Since EGF is mitogenic for PC12 cells and differentiation may not occur until proliferation is inhibited, differentiation caused by EGF and cAMP may be due to the antiproliferative activity of cAMP. To test this hypothesis, we examined the effect of EGF or combinations of EGF and cAMP on PC12 cell proliferation. EGF alone stimulated proliferation of PC12 cells and increased the levels of several cell cycle progression factors including cdk2, cdk4, and cyclin B1. Cyclic AMP inhibited the EGF-stimulated increases in cell cycle progression factors as well as proliferation. Other antiproliferative agents including rapamycin, mimosine, and nitric oxide agonists also synergized with EGF to stimulate differentiation. These data indicate that the coupling of antiproliferative signals with EGF modifies the biological properties of EGF and converts it to a differentiating growth factor.

Neuronal differentiation of PC12 cells by nerve growth factor (NGF)1 is characterized by several changes including growth arrest, activation of specific genes, and neuritogenesis. A critical step for differentiation is arrest of the cell cycle. Although NGF treatment initially stimulates proliferation, this is followed by cessation of DNA synthesis and cell division (1–4). In addition, an asynchronous population of PC12 cells accumulates in a G1-like state following NGF treatment (5, 6). These findings imply that the cessation of cell proliferation in G1 may be crucial for neuronal differentiation. Although the mechanism by which NGF arrests PC12 cells in G1 phase is not fully understood, there appears to be a direct correlation between the differential regulation of specific cell cycle regulatory components and NGF-induced differentiation (1, 7).

EGF- and NGF-treated PC12 cells share several common properties, including activation of the MAP kinase pathway, induction of immediate early genes (IEGs), membrane ruffling, and increased cell adhesion (8–12). In contrast to NGF, EGF is a potent mitogen that does not stimulate differentiation (13). However, the molecular mechanisms differentiating these two growth factors are not fully understood. EGF, in combination with low levels of cAMP (50% increases above basal), stimulates differentiation of PC12 cells (14), whereas cAMP alone does not. Differentiation stimulated by EGF and cAMP is blocked by inhibitors of transcription as well as inhibitors of the MAP kinase and protein kinase A signal transduction systems indicating that activation of both pathways is required. This is particularly interesting since it suggests that pairing of two signals, one generated through the cAMP pathway and the other via the MAP kinase pathway, stimulates neuronal differentiation. The purpose of this study was to determine if cAMP inhibits increases in DNA synthesis and proliferation caused by EGF. Our data indicate that physiologically relevant cAMP signals and other antiproliferative agents synergize with EGF to differentiate PC12 cells.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal cyclin B1, polyclonal cdk2, and polyclonal cdk4 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies used were either goat anti-mouse IgG-horseradish peroxidase (HRP) conjugated from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) or goat anti-rabbit IgG-HRP conjugated from Life Technologies, Inc. (Grand Island, NY).

PC12 Cell Culture—PC12 cells were maintained at 37 °C in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin and streptomycin, 5% bovine calf serum, and 10% fetal bovine serum in a humidified 95% air, 5% CO2 incubator. Cells were subcultured onto poly-L-lysine (50 mg/ml) coated tissue culture dishes. When the cultures reached 20–30% confluency, cells were treated with various effector agents for 24–48 h after plating.

MAP Kinase Assays—MAP kinase was assayed by the general method of Gotot et al (9) using myelin basic protein as a substrate. A mouse anti-MAP kinase monoclonal antibody was purchased from Zymed Laboratories. PC12 cells were incubated with 50 ng/ml NGF, 50 ng/ml EGF, or 50 ng/ml EGF + 50 μg dibutyryl-cAMP for varying periods of time as indicated. Cells extracts were prepared for MAP kinase immunoprecipitation and kinase reactions in SDS-polyacrylamide gels were carried out as described in the product information bulletin from Zymed Laboratories. Gels were incubated at room temperature for 1 h in 10 ml of 40 mM Hepes, pH 8.0, 2 mM dithiothreitol, 100 μM EGTA, 5 mM MgCl2, 25 μM ATP with [γ-32P]ATP (250 μCi; 1 μCi = 37 kBq). The gels were vigorously washed in 5% trichloroacetic acid, 1% NaPPi, dried, and exposed to x-ray film for 10–12 h. MAP kinase activities are expressed as relative activities determined by scanning the exposed film with a Bio-Rad GS-670 densitometer.

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Quantitation of Neurite Outgrowth—Treatment of PC12 cells with cAMP alone causes transient neurite outgrowth that is not dependent upon transcription. In a previous study, we established that neurite outgrowth stimulated by combinations of EGF and cAMP measured after 14 days is nontransient and dependent upon transcription (14). Therefore, neurite outgrowth was monitored as an indicator of PC12 differentiation. Cells were treated with various effectors at the indicated concentrations for 14 days. Cells were photographed, and neurite lengths were quantitated from photographs of cells using a ruler. Values for neurite lengths are an average from three independent experiments ± S.D. (n = 200 cells).

Determination of Total Cell Number—Cells were treated with various effectors and harvested every odd day for 11 days. Harvested cells were diluted, and total cell number was counted on a hemacytometer. Values for total cell number are expressed as percentage change compared with untreated cells from one representative experiment. At least three independent experiments were performed for each set of conditions.

Measurement of $[^3H]$Thymidine Incorporation—Cells were treated with various effectors in triplicate at the indicated concentrations. PC12 cells were then pulse-labeled with 2 nCi/ml $[^3H]$thymidine in fresh Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin and streptomycin, 5% bovine calf serum, and 10% fetal bovine serum for 2 h at 37°C in a humidified 95% air, 5% CO$_2$ incubator. After 2 h, dishes were rinsed twice with 5% trichloroacetic acid at 4°C and drained. Cells were lysed in 1 ml of 0.25 M NaOH for 10 min at room temperature on a platform shaker. Samples were then transferred to scintillation vials containing 10 ml of EcoLite and counted for $[^3H]$thymidine incorporation. Cells were harvested every odd day for 11 days. Values for $[^3H]$thymidine incorporation are expressed as a percentage change compared with untreated cells from one representative experiment. At least three independent experiments were performed.

Determination of Changes in Cell Cycle Proteins—Cells were treated with various effectors at the indicated concentrations for 0, 1, 3, 6, or 9 days. They were harvested by scraping dishes and were sonicated with various effectors at the indicated concentrations for 0, 1, 3, 6, or 9 days. PC12 cells were then pulse-labeled with 2 nCi/ml $[^3H]$thymidine in fresh Dulbecco’s modified Eagle’s medium supplemented with 1% penicillin and streptomycin, 5% bovine calf serum, and 10% fetal bovine serum for 2 h at 37°C in a humidified 95% air, 5% CO$_2$ incubator. After 2 h, dishes were rinsed twice with 5% trichloroacetic acid at 4°C and drained. Cells were lysed in 1 ml of 0.25 M NaOH for 10 min at room temperature on a platform shaker. Samples were then transferred to scintillation vials containing 10 ml of EcoLite and counted for $[^3H]$thymidine incorporation. Cells were harvested every odd day for 11 days. Values for $[^3H]$thymidine incorporation are expressed as a percentage change compared with untreated cells from one representative experiment. At least three independent experiments were performed.

RESULTS

Low Levels of cAMP Do Not Stimulate MAP Kinase—In a previous report, we discovered that pairing of low cAMP signals with EGF stimulated differentiation of PC 12 cells. The increases in cAMP required for this effect were only 50–80% above basal cAMP. cAMP increases of this magnitude are generated with 50 μM dibutyrly-cAMP or 0.1 μM forskolin. Since NGF differentiates PC12 cells and stimulates a larger increase in MAP kinase activity than EGF, it might be argued that pairing low levels of cAMP with EGF causes differentiation because cAMP enhances MAP kinase activity. To address this issue, we monitored MAP kinase activity in the presence of EGF, EGF + 50 μM dibutyrly-cAMP, or NGF (Fig. 1). As expected, NGF caused a greater and more prolonged increase in MAP kinase activity than EGF alone. EGF + 50 μM dibutyrly did not stimulate MAP kinase activity beyond that seen with EGF alone. Therefore, differentiation caused by EGF and cAMP is not due to increases in MAP kinase activity above that caused by EGF.

cAMP Inhibits EGF-stimulated Proliferation—To determine if proliferation of PC12 cells stimulated by EGF is inhibited by increases in intracellular cAMP, we quantitated proliferation and $[^3H]$thymidine incorporation after treatment with EGF, or EGF with cAMP-elevating agents including forskolin and cholera toxin. Forskolin and cholera toxin increase intracellular cAMP by direct activation of adenyl cyclase or activation of G$_s$, respectively. The effects of growth factors or cAMP on cell number and $[^3H]$thymidine incorporation are expressed as increases relative to untreated controls. Treatment of PC12 cells with 0.5 μM forskolin, 50 μM dibutyrly-cAMP, or 50 ng/ml cholera toxin increases cAMP approximately 50–80% above basal (14). Forskolin or cholera toxin reduced proliferation by 46 and 63%, respectively (Fig. 2, B and C). As expected, EGF stimulated proliferation. The number of cells increased approximately 300% after 9 days of treatment. EGF-stimulated proliferation was almost completely inhibited by all three cAMP elevating agents. These data indicate that relatively small cAMP increases inhibit EGF-stimulated proliferation.

Another indicator of growth arrest is a reduction in DNA synthesis. To determine the relative changes in DNA synthesis, $[^3H]$thymidine incorporation was monitored after treatment with EGF or EGF with cAMP. EGF increased $[^3H]$thymidine incorporation approximately 228% by day 11 of treatment (Fig. 3). Forskolin, or cholera toxin alone did not cause significant changes in $[^3H]$thymidine incorporation relative to untreated cells (Fig. 3, B and C). However, these agents inhibited the increase in $[^3H]$thymidine incorporation caused by EGF.

Synergistic Induction of Differentiation by EGF and Antiproliferative Agents—If stimulation of differentiation by combinations of EGF and cAMP is attributable to the antiproliferative activity of cAMP, then other antiproliferative agents should also differentiate PC12 cells when administered with EGF. To test this hypothesis, PC12 cells were treated with EGF plus rapamycin, mimosine, aphidicolin, or nitric oxide (NO) agonists SIN-1, SNAP, and SNP. Mimosine and aphidicolin arrest cells in G$_1$ by inhibiting ribonucleotide reductase and DNA polymerase activity, respectively. Rapamycin causes growth cessa-
Fig. 2. cAMP inhibits proliferation of PC12 cells stimulated by EGF. A, the number of PC12 cells was quantitated at the indicated times in culture with no treatment (control), 50 ng/ml EGF, 50 μM dibutyryl-cAMP, or 50 ng/ml EGF + 50 μM dibutyryl-cAMP. B, the number of PC12 cells was quantitated after treatment with 50 ng/ml EGF, 0.5 μM forskolin, or 50 ng/ml EGF + 0.5 μM forskolin. C, the number of PC12 cells was counted after treatment with 50 ng/ml EGF, 50 ng/ml cholera toxin, or 50 ng/ml EGF + 50 ng/ml cholera toxin. D, cell number was quantitated after treatment with 20 ng/NGF, 50 μM dibutyryl-cAMP, or 20 ng/NGF + 50 μM dibutyryl-cAMP. Intracellular cAMP levels were increased 50% over basal by 0.5 μM forskolin or 50 ng/ml cholera toxin. Cell number was quantitated using a hemacytometer and is expressed as percent change relative to untreated cells. Representative data from one of three independent experiments are reported.

Fig. 3. cAMP inhibits DNA synthesis in PC12 cells stimulated by EGF. A, incorporation of [3H]thymidine was determined at the indicated times in culture with no treatment (control), 50 ng/ml EGF, 50 μM dibutyryl-cAMP, or 50 ng/ml EGF + 50 μM dibutyryl-cAMP. B, [3H]thymidine incorporation was determined after treatment with 50 ng/ml EGF, 0.5 μM forskolin, or 50 ng/ml EGF + 0.5 μM forskolin. C, [3H]thymidine incorporation was determined after treatment with 50 ng/ml EGF, 50 ng/ml cholera toxin, or 50 ng/ml EGF + 50 ng/ml cholera toxin. D, [3H]thymidine incorporation was determined after treatment with 20 ng/NGF, 50 μM dibutyryl-cAMP, or 20 ng/NGF + 50 μM dibutyryl-cAMP. The levels of [3H]thymidine incorporation were quantitated as described under “Experimental Procedures” and expressed as a percent change relative to untreated cells. Representative data from one of three independent experiments are reported.

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Inhibition of EGF-stimulated Expression of cdk2, cdk4, and Cyclin B1 by cAMP—NGF causes a gradual decrease in protein levels of cdc2, cdk2, cdk4, and cdk6 in PC12 cells as well as decreases in cyclin B1 (7). The correlation between NGF-stimulated differentiation and a decrease in regulatory components of the cell cycle suggests that NGF may decrease proliferation of PC12 cells by controlling the expression of these proteins. Since EGF and antiproliferative agents induce differentiation, we quantitated the expression of several cell cycle regulatory proteins important for G1/S and G2/M transitions. The expression of cdk2, cdk4, and cyclin B1 was examined after exposure to EGF or EGF with cAMP. As with previous experiments, the concentrations of forskolin, cholera toxin, or dibutyryl-cAMP used increased intracellular cAMP by 50%.

EGF-treated cells displayed a significant increase in cdk2 expression, reaching a maximum of +694% (Fig. 6). This is consistent with induction of proliferation by EGF and the fact that cdk2 is crucial for progression from G1 to S phase (17–20). Increases in intracellular cAMP inhibited EGF-stimulated cdk2 increases. Similar results were obtained when cdk4 expression was analyzed. EGF caused a marked increase in cdk4 that was blocked by dibutyryl-cAMP, forskolin, or cholera toxin (Fig. 7).

Cyclin B1 is a key regulatory component for the G2/M phase transition in the cell cycle. An induction of cyclin B1 in S/G2 followed by cAMP-mediated degradation of cyclin B is necessary for the completion of mitosis (21). Therefore, cyclin B1 expression was also examined after treatment with EGF. EGF treatment of PC12 cells increased cyclin B1 expression by approximately 147% above untreated cells (Fig. 8). Dibutyryl-cAMP, forskolin, and cholera toxin also caused a transient elevation in cyclin B1 expression that subsequently decreased after several days (Fig. 8). A similar pattern of cyclin B1 expression was also evident when EGF was paired with cAMP elevating agents. There was an initial rise in cyclin B1 expression that decreased below basal levels 6 days after addition of EGF and cAMP elevating agents. This initial rise in cyclin B may be caused by EGF-stimulated growth through G1/S phase transitions followed by cAMP-mediated degradation of cyclin B during mitosis (21).

**DISCUSSION**

In a previous study, we reported that EGF in combination with low levels of cAMP stimulates differentiation of PC12 cells (14). The cAMP increases necessary to convert EGF to a differentiating agent are well within normal physiological limits, approximately 50% over basal. Neither EGF alone nor cAMP at low levels promotes differentiation. This phenomenon is dependent upon activation of the MAP kinase pathway coupled with stimulation of the cAMP signal transduction system (14).
We hypothesize that the antiproliferative activity of cAMP may contribute to this phenomenon because cells must stop dividing before they can differentiate. The objectives of this study were to determine if low level cAMP signals inhibit EGF-stimulated proliferation, to determine if cAMP signals of this magnitude affect the expression of proteins important for cell cycle progression, and to determine if other antiproliferative agents act with EGF to stimulate differentiation.

High levels of cAMP, generated by 10–100 µM forskolin, cause transient neurite outgrowth. However, neurites produced under these conditions are not stable, and the cells are not permanently differentiated. It is also unlikely that cAMP increases produced by 10–100 µM forskolin are physiologically relevant; they exceed cAMP increases produced by neurotransmitters, Ca^{2+}, or combinations of effectors. Activation of the cAMP signal transduction system alone is not sufficient to cause differentiation of PC12 cells. The changes produced by high cAMP are not dependent upon transcription and are transient.

EGF stimulated increases in cell number, DNA synthesis, and several cell cycle progression factors that were inhibited by cAMP. The concentrations of these agents used were selected to generate cAMP increases approximately 50% above basal since we had previously shown that cAMP signals of this magnitude will differentiate PC12 cells treated with EGF (14). These data suggest that the antiproliferative activity of cAMP may contribute to differentiation caused by cAMP + EGF. If this hypothesis is valid, then other antiproliferative agents might also...
synergize with EGF to stimulate differentiation. Indeed, several antimitotic agents including rapamycin, mimosine, aphidicolin and NO-generating agents differentiated PC12 cells in combination with EGF.

Other data in the literature support the general hypothesis that inhibition of proliferation must precede neuronal differentiation. Development of the mammalian embryonic nervous system involves an initial burst of cell division. Neurons then become terminally postmitotic, differentiate, and form postsynaptic connections. Interestingly, PC12 cells behave in a similar manner when treated with NGF. There is an initial proliferation of cells, followed by a cessation of growth, and then cells differentiate. NGF functions as a progression factor, possessing both mitogenic and antimitogenic activities (6). The antimitogenic activity of NGF is mediated by NO; treatment of PC12 cells with NGF induces the expression of nitric oxide synthase (16). Cessation of proliferation appears to be a prerequisite for neuronal differentiation in NGF-treated PC12 cells and does not occur when cells are treated with EGF alone. EGF is a known mitogen for PC12 cells (22), whereas cAMP is antimitogenic, inhibits DNA synthesis, and accumulates cells in G2/M phase (23–26).

Growth arrest during neuronal differentiation may be due to changes in expression of several key cell cycle proteins. The
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temporal correlation between cessation of proliferation, down-regulation of cell cycle regulatory protein expression, and neuronal differentiation supports this hypothesis. NGF-induced differentiation of PC12 cells decreases the expression of cdc2, cdk2, cdk4, cdk6, and cyclin B1 (1, 7). A similar depression in cdc2 and cdk2 expression occurs in terminally differentiated N1E-115 cells (27). We also observed a down-regulation of cdc2, cdk4, and cyclin B1 when PC12 cells were treated with EGF + cAMP. Cdk2 activity is essential for the progression from G1 to S phase (17–19, 28, 29), and overexpression of cdk2 inhibits neuronal differentiation (30). Since cAMP inhibited EGF-stimulated expression of cdk2, this may explain why cAMP inhibits the growth of EGF-treated cells. Similarly, cyclin B has been implicated in the G2/M transition because of its expression at this stage of the cell cycle (29, 31).

Cyclic AMP modulates the expression of several key regulatory components of the cell cycle. An increase in expression of cyclin B followed by cAMP/protein kinase A-mediated degradation is crucial for the progression of cells from mitosis to interphase (21). Inhibition of protein kinase A by overexpression of the regulatory subunit during the onset of M phase results in growth arrest of cells during mitosis. However, growth arrest is reversed following the addition of cAMP enhancing agents. EGF + cAMP-induced neuronal differentiation resulted in an initial rise in cyclin B1 expression that decreased below basal levels by day 6 in PC12 cells. This initial rise in cyclin B may be caused by EGF-stimulated growth through G1/S phase transition followed by cAMP-mediated degradation of cyclin B during mitosis (21). Furthermore, CAMP blocks the mitogenic effects of colony-stimulating factor 1 (CSF-1) by increasing expression of p27Kip1, a cyclin D/cdk4 inhibitor, thus inducing mid-G1 phase growth arrest in macrophages (32). Similar effects have also been seen in various cell lines following growth factor stimulation (33, 34). In addition, CAMP inhibits growth factor-stimulated expression of G1 phase, cyclins D1 and E, and the activation of cyclin A- and cyclin E-dependent histone H1 kinases (33, 35, 36). Cyclic AMP induces G1 arrest, increases the critical cell size required for budding, and inhibits the expression of G1 phase cyclins, CLN1 and CLN2, in yeast. Mutations inactivating the regulatory subunit of protein kinase A or the cAMP-dependent phosphodiesterase cause G1 arrest that is reversed by inducing expression of CLN1 and CLN2 genes in yeast (37–39). Collectively, these studies support the general hypothesis that cAMP-induced inhibition of cell cycle regulatory proteins may contribute to differentiation stimulated by EGF + cAMP.

Our data indicate that CAMP in combination with EGF induces growth arrest, inhibits DNA synthesis, and promotes neuronal differentiation of PC12 cells. The temporal correlation between growth arrest induced by combinations of EGF and cAMP, depression of regulatory cell cycle components, and neuronal differentiation suggests that the antimitotic activity of cAMP contributes to this phenomena. In summary, pairing of antiproliferative signals with EGF modifies the biological properties of EGF and converts it to a differentiating growth factor.

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