Differential and Synergistic Actions of Nerve Growth Factor and Cyclic AMP in PC12 Cells

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ABSTRACT When a clonal line of rat pheochromocytoma (PC12) was exposed to $\beta$-nerve growth factor (NGF), N$^6$,O$^2$-dibutyryl adenosine 3':5' cyclic monophosphate (Bt$\beta$cAMP), or a combination of the two, 10, 26, or 70% of the cell clumps, respectively, displayed neurites after 1 d. Increases in the cellular RNA concentration were also found to be additive or greater when both agents were present. Neurites induced by Bt$\beta$cAMP alone were not maintained after replacement with NGF. The degree of potentiated neurite outgrowth was a function of the time of simultaneous exposure to both agents. The initiation of neurite outgrowth in the presence of Bt$\beta$cAMP was independent of RNA synthesis, in contrast to that induced by NGF alone. We conclude that NGF-induced initiation of morphological differentiation of these cells is not mediated by a cAMP-dependent mechanism. Consideration of Bt$\beta$cAMP effects upon other cell lines suggests that Bt$\beta$cAMP causes a rapid, but unstable, reorganization of the PC12 cytoskeleton, resulting in the initiation of neurite outgrowth from these cells. In contrast, NGF alone achieves a more stable cytoskeleton reorganization by an RNA synthesis-dependent mechanism.

Nerve growth factor (NGF) is a polypeptide hormone that is involved in the development and maintenance of sympathetic and certain sensory neurons (12). Recently, a pheochromocytoma clonal cell line, PC12, showing similar NGF-responsive properties, has been isolated (5). When PC12 cells are exposed to NGF, they extend neuritic processes and cease cell division within 1 wk (5). NGF also prevents the death of these cells and stimulates their differentiation in serum-free medium (4). The induction of differentiation is RNA synthesis dependent (1). However, the regeneration of neuritic processes from cells that have been exposed to NGF for a week does not require RNA synthesis (1). These results have led to the suggestion that the synthesis of new RNA species and possibly proteins is required for the initiation of neurite outgrowth. However, once these products are made and initiation has occurred, regeneration of processes over a 24-h period can occur independent of further RNA synthesis (1). The nature and role of these gene products are unknown.

Two suggestions for the mechanisms responsible for the initiation of neurite outgrowth have been made. It has been proposed that cAMP is the second messenger in the response of PC12 cells to NGF (16–18). This proposal is based on the observations that NGF increases the cAMP content of PC12 cells during the first minutes of exposure (18); that cAMP initiates neurite outgrowth from these cells (16, 17), and that two analogs of cAMP modulate the synthesis of the same group of proteins in PC12 cells, as does NGF (2). The suggestion for an alternative mechanism comes from the finding of specific NGF receptors on the nuclear membrane of PC12 cells, and of a time- and temperature-dependent translocation of NGF (25) from its surface receptors (9, 25) to those on the nucleus (25). The association of NGF with the nucleus is necessary for both the initiation and the regeneration of neurite outgrowth from PC12 cells.\(^1\)

We have further examined the effect of N$^6$,O$^2$-dibutyryl adenosine 3':5' cyclic monophosphate (Bt$\beta$cAMP) upon PC12 cells. Studies at both a biochemical and morphological level indicate that NGF and cAMP act by separate mechanisms and together produce synergistic effects. In particular, initiation of neurite outgrowth from these cells in the presence of both agents is RNA- and protein-synthesis independent. It is suggested that Bt$\beta$cAMP causes a rapid reorganization of the cytoskeleton that NGF alone achieves by an RNA synthesis-dependent mechanism.

\(^1\)Yankner, B. A., and E. M. Shooter. Manuscript submitted.
MATERIALS AND METHODS

Cell Culture

The PC12 clone of a rat pheochromocytoma was obtained from David Schubert (Salk Institute, La Jolla, Calif.). The cells were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.), containing 10% fetal calf serum and 5% horse serum on polystyrene tissue culture dishes (NUNC, Irvine Scientific, Calif.). Cultures were maintained in a water-saturated atmosphere of 88% air/12% CO₂. Experiments were performed by replating subconfluent cells at a density of 1-2 x 10⁵ cells/cm², and the cells were allowed to reach log phase growth (2 d). At that time, fresh medium was added, together with 5 ng/ml βNGF (prepared by the method of Smith et al. [21]) or 1 mM Bt₂cAMP (Sigma Chemical Co., St. Louis, Mo.), unless otherwise indicated. In some experiments, the fresh medium added contained 1 mg/ml bovine serum albumin (BSA, Pentex, Miles Biochemicals, Ind.), with or without fetal calf and horse serum. It has been found that, for all parameters measured in this study, maximum stimulation is attained at concentrations equal to or below 5 ng/ml for βNGF and 1 mM for Bt₂cAMP.²

The morphological differentiation of the cells was assayed by determining the percentage of cell clumps with neuritic processes longer than one cell body diameter and displaying a growth cone at their tip. Clumps, rather than single cells, were scored because addition of βNGF leads to extensive clumping of these cells (see reference 3). At least 400 clumps were examined in randomly chosen fields on each plate. All experiments were performed at least three times with similar results.

Inhibition of RNA and Protein Synthesis

Actinomycin-D (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) and cycloheximide (Sigma Chemical Co.) were added to cultures at 0.5 μM and 10 μg/ml, respectively. 1 h later, βNGF and Bt₂cAMP were added along with [3H]uridine (1 μCi/ml) or [3H]proline (2 μCi/ml) (Amersham Corp., Arlington Heights, Ill., sp. act. 27 and 18 Ci/mmole, respectively). After 3 h, five randomly chosen fields on each plate were photographed, and the cells were immediately harvested and assayed for RNA and protein synthesis (1). Uptake into the acid-soluble compartment was unaffected by the drugs. Incorporation of [3H]uridine into RNA was inhibited >97% by 0.5 μM actinomycin-D. Cycloheximide at 10 μg/ml inhibited [3H]proline incorporation into protein by >97%. Uptake and incorporation were measured relative to control sister cultures that received neither drug.

DNA, RNA, and Protein Determinations

The cells were mechanically digested from the dishes by trituration and collected by centrifugation at 750 g, for 5 min. Acid-soluble material was removed by extracting the cell pellet three times with 0.5 M perchloric acid (PCA) at 4°C. The acid-insoluble material was suspended in 1 M NaOH and incubated at 37°C for 18 h. A sample of the solution was taken for protein determination (11), using bovine serum albumin (Sigma Chemical Co.) as a standard. Concentrated PCA was added to the remainder of the solution, to a final concentration of 0.5 M PCA and left at 4°C for 1 h. The resulting precipitate was collected at 10,000 g, for 10 min and washed once with 0.5 M PCA. The two supernates were combined for RNA determination (22). The pellet was extracted twice with 0.5 M PCA at 80°C for 30 min, and the combined supernates were taken for DNA determination (22). The ratios of RNA/DNA and protein/DNA were taken as a measure of the cellular RNA and protein concentrations.

RESULTS

Potentiation of Morphological Differentiation

Exposure of PC12 cells to βNGF for 24 h produces little morphological change except for a tendency to flatten out on the polystyrene substrate (Fig. 1A, and B). The cells respond to Bt₂cAMP by extending short neurites (Fig. 1C). However, βNGF in combination with Bt₂cAMP produces a morphological effect far greater than the individual effects of these agents (Fig. 1 D). The combined treatment produces a morphological differentiation normally seen only after 4-5 d of exposure to βNGF.

² Gunning, P. W., G. E. Landreth, P. Layer, M. Ignatius, and E. M. Shooter. J. Neurosci. In press.

This potentiation of neurite outgrowth depends upon the βNGF concentration. In the presence of Bt₂cAMP only, ~25% of the cell clumps possess neurites after 24 h. Addition of βNGF at increasing concentrations raises this to a maximum of 70% at 0.5-1.0 ng/ml βNGF (Fig. 2). The dose response of this potentiation by βNGF is identical to the dose-response curve for βNGF-promoted neurite regeneration from differentiated PC12 cells (3, 4).

The specificity of the Bt₂cAMP effect was evaluated by repeating the experiments described above but 2 mM Na butyrate, 1 mM Bt₂cAMP, or 1 mM AMP was substituted for Bt₂cAMP. Neurite outgrowth was not observed when these agents alone were used, and none of them potentiated the βNGF-induced response. These results indicate a specificity for the cAMP moiety of Bt₂cAMP and suggest the involvement of a cAMP-dependent mechanism. This is supported by the observation that cholera toxin, which increases cAMP levels within mammalian cells (10), produced a neurite outgrowth and βNGF potentiation similar to that observed using Bt₂cAMP (Fig. 3).

Effects on Cellular RNA Concentration

The PC12 cellular RNA concentration is increased within 24 h of exposure to βNGF, the increase being half the maximum at ~0.25 ng/ml βNGF, and the maximum between 2.5 and 5.0 ng/ml.³ We, therefore, examined the effects of Bt₂cAMP on cellular RNA levels with and without βNGF. After 24 h of exposure to βNGF, in medium containing either serum or BSA, the RNA concentration increased 20% over controls (Table I). Treatment with Bt₂cAMP also increased the RNA concentration in both media. This effect was maximum at 3 x 10⁻⁴ to 10⁻³ M Bt₂cAMP. However, in medium containing serum, Bt₂cAMP produced a significantly greater stimulation than βNGF, whereas the converse was true in medium containing only BSA (Table I). The poor effect of Bt₂cAMP in medium plus BSA was due to a lack of serum rather than inhibition by BSA, because the addition of serum to medium plus BSA restored the full Bt₂cAMP stimulation (Table I). Thus, although the magnitude of βNGF stimulation is independent of the culture medium, that of Bt₂cAMP is dependent, at least in part, upon the presence of serum.

Exposure of the cells to both Bt₂cAMP and βNGF produced an increase in RNA equal to the sum of the increases separately induced by each agent when examined in medium plus serum. Using medium plus BSA, the combination produced a greater than additive increase (Table I). This result is inconsistent with a common mechanism of action for both agents, because maximally stimulating concentrations of both agents together produce an additive, or greater than additive, effect. Similar results were obtained when the PC12 cellular protein concentrations were measured (not shown).

Both the morphological and biochemical data suggest that the actions of βNGF and Bt₂cAMP are complementary and, in some situations, synergistic.

Potentiation Requires Simultaneous Presence of βNGF and Bt₂cAMP

When PC12 cells are exposed to βNGF for several days and then replated with fresh medium plus βNGF, they regenerate their neurites within 24 h (3); such cells are said to be "primed." In contrast, when cells exposed to Bt₂cAMP for several days are replated in the presence of βNGF, they do not regenerate...
FIGURE 1 Potentiation of neurite outgrowth by NGF plus Bt2cAMP. Long phase PC12 cells were exposed to βNGF (5 ng/ml), Bt2cAMP (1 mM), or both for 24 h, and then photographed. A, control; B, βNGF; C, Bt2cAMP; D, βNGF plus Bt2cAMP. Bar, 50 μm.

their neurites rapidly (G. E. Landreth, P. W. Gunning, and E. M. Shooter, unpublished observations). This suggests that the Bt2cAMP-treated cells, although displaying significant neurite initiation, are not “primed” in a manner analogous to that produced by βNGF (4). Therefore, we investigated the stability of the Bt2cAMP effect by pretreating the cells with Bt2cAMP, and then adding βNGF in the continued presence or absence of Bt2cAMP.

If cells were treated with Bt2cAMP for 8 h, followed by exposure to βNGF for 16 h, virtually no neurite outgrowth was observed (Table II). However, the presence of βNGF plus Bt2cAMP for the final 16 h resulted in maximum neurite outgrowth. An identical result was obtained when βNGF was present for the first 8 h, followed by both agents for the remainder of the 24-h period (Table II). Repeating this experiment with a 20-h exposure to Bt2cAMP, followed by 4 h with βNGF alone, resulted in virtually no neurite outgrowth (Table II). After a 20-h treatment with only Bt2cAMP, ~25% of the cell clumps had neurites. It is therefore obvious that βNGF alone cannot maintain neurites induced by Bt2cAMP. In contrast, if βNGF and Bt2cAMP are present for the final 4 h, a considerable potentiation of neurite outgrowth is observed. Pre-exposure to βNGF for 20 h, followed by the simultaneous presence of βNGF and Bt2cAMP for the final 4 h, gave a

FIGURE 2 Relationship of βNGF concentration to potentiation of neurite outgrowth. Log phase PC12 cells were exposed to 1 mM Bt2cAMP plus various concentrations of βNGF for 24 h. The resulting neurite outgrowth was determined.
Fresh medium plus serum or BSA was added to log phase PC12 cells, together with Bt2cAMP (1 mM), PNGF (5 or 50 ng/ml), and BSA (1 mg/ml) in the appropriate plates. After 24 h, the cells were harvested and the cellular RNA concentration was measured. The values are expressed relative to control sister cultures as mean ± SD. The number of individual cultures assayed is given in parentheses. The significance of differences observed was determined from Student’s t-test for two means. Values with the same superscript were not significantly different (NS). The salient P-values obtained were: a vs. b, P < 0.00005; a vs. d, NS; d vs. e, P < 0.02; d* vs. e, P < 0.001; b vs. e, P < 0.00005; b vs. c, P < 0.00005; e vs. f, P < 0.00005; c vs. f, NS.

**TABLE I**

| Additions                  | Medium and serum | Medium and BSA |
|----------------------------|------------------|----------------|
|                            | 5                | 50             | 5              | 50             |
| βNGF                      | 1.21 ± 0.05 (15) | 1.22 ± 0.05 (16)| 1.19 ± 0.04 (6) | 1.23 ± 0.05 (6) |
| Bt2cAMP                   | 1.36 ± 0.04 (9)  |                | 1.14 ± 0.03 (10)|                |
| βNGF + Bt2cAMP            | 1.58 ± 0.06 (6)  | 1.53 ± 0.08 (3) | 1.48 ± 0.04 (6) | 1.50 ± 0.03 (6) |
| Bt2cAMP + BSA             | 1.34 ± 0.01 (2)  |                |                |                |
| βNGF + Bt2cAMP + BSA      | 1.51 ± 0.04 (2)  |                |                |                |

Various additions and substitutions of 1 mM Bt2cAMP and 5 ng/ml βNGF were made over a 24 h period to log phase cells in medium containing serum. After 24 h, the percentage of cell clumps displaying neurites was determined ranging from 1 nM to 10 μM, and then βNGF and Bt2cAMP were added. After 1 h, 10% (±2%) of the cells displayed neurites independent of the presence of the inhibitor at any of these concentrations (not shown). In another experiment, the cells were exposed to cycloheximide or actinomycin-D for 1 h before the addition of βNGF plus Bt2cAMP. Neurite outgrowth examined after 3 h was not inhibited under these conditions, whereas RNA synthesis was inhibited >80% by actinomycin-D, and protein synthesis was inhibited >97% by cycloheximide (Fig. 4). Thus, initiation of neurite outgrowth induced by βNGF plus Bt2cAMP is independent of transcription and translation.

This conclusion is supported by recent experiments performed using scanning electron microscopy, rather than phase-contrast light microscopy, to evaluate neurite outgrowth. We noted that Bt2cAMP plus βNGF caused a significant level of neurite outgrowth within 2 min of their addition to cells. Such a rapid response is inconsistent with any transcriptional or translational involvement.

**Effects of Cytoskeletal and DNA Synthesis Inhibitors**

DNA synthesis within PC12 cells is inhibited by addition of Bt2cAMP (M. A. Bothwell, unpublished observation). To de-
termine whether inhibition of DNA synthesis could cause neurite outgrowth and a potentiation of βNGF-induced morphogenesis, cells were exposed to 1 μM cytosine arabinoside (Sigma Chemical Co.) in both the presence and absence of βNGF. Although DNA synthesis was inhibited by >99%, no neurite outgrowth was observed with the inhibitor alone, and no potentiation was observed after a 24-h coexposure with βNGF. In contrast, when 0.5 μg/ml colchicine (Sigma Chemical Co.) or 4 μM cytochalasin B (Sigma Chemical Co.) was added to the cells, along with βNGF plus Bt2cAMP, no neurite outgrowth was observed at any time.

**DISCUSSION**

The results presented in this paper lead to the following conclusions: (a) Bt2cAMP and βNGF have a synergistic effect upon the initiation of neurite outgrowth from PC12 cells; (b) Bt2cAMP and βNGF have additive and synergistic effects upon the RNA levels within PC12 cells; (c) βNGF cannot
maintain βt2cAMP-induced morphogenesis; (d) the potentiation of neurite outgrowth by βt2cAMP plus βNGF requires the simultaneous presence of both agents; and (e) the induction of de novo neurite outgrowth by βt2cAMP plus βNGF is independent of ongoing transcription, in contrast to that induced by βNGF alone. Therefore, it is clear that βt2cAMP and βNGF have complementary effects on this cell line, together producing a greatly enhanced response over that observed with either agent alone, and that they act to initiate neurite outgrowth by different mechanisms. This is made particularly clear by the observation that neurite outgrowth can be initiated by βt2cAMP plus βNGF, but not by βNGF alone, under conditions where RNA synthesis is largely abolished. Thus, cAMP can overcome the transcription dependency for βNGF-induced neurite outgrowth in a manner analogous to the way in which βNGF can “prime” PC 12 cells for transcription-independent neurite regeneration. Because the cAMP-mediated increase in RNA levels is largely dependent on the presence of serum in the medium, whereas the NGF-mediated increase is not, it is suggested that the mechanisms leading to elevation of cellular RNA by these two agents are also distinct.

Even if βNGF is added afterward, the neurites produced by βt2cAMP are unstable, in the sense that they are rapidly lost on removal of βt2cAMP. Thus, it is unlikely that a βNGF-induced, transient rise in cAMP levels can by itself lead to neurite initiation through a cAMP-dependent step originating with cAMP as a second messenger. Instead, rapidly reversible effects of cAMP on the morphology of PC 12 cells are consistent with rapidly reversible morphological and biochemical effects of cAMP described in other systems (7, 13, 14). It should also be noted that Hatanaka et al. (6) failed to detect any transient increase in the cAMP content of PC 12 cells after interaction with NGF, and that Landreth et al. (8), unlike Schubert et al. (17), found no significant change in calcium efflux from NGF-treated PC 17 (12).

This conclusion is supported by the observation that if βNGF is removed from the culture medium by adding anti-βNGF antibody any time during the first 3–4 h of exposure, no neurite outgrowth occurs from PC 12 cells (see footnote 1). This is consistent with a recent paper by Hatanaka et al. (17) showing that when βt2cAMP is removed from the culture medium, the neurites do not survive more than 16 h if the medium is not replaced by fresh medium containing βt2cAMP. Similar rapidly reversible effects of βt2cAMP upon Chinese hamster ovary cell morphology have been shown to be related to induction of microtubule and microfilament organization (7, 13, 14). Finally, the induction of neurite outgrowth by βt2cAMP in the presence and absence of βNGF is rapidly reversed upon removal of βt2cAMP from the medium. Similar rapidly reversible effects of βt2cAMP upon Chinese hamster ovary cell morphology have been shown to be related to induction of microtubule and microfilament organization (7, 13, 14). Finally, the induction of neurites in the presence of βt2cAMP is RNA and protein synthesis independent. Similarly, βt2cAMP-induced changes in cell morphology and organization of the cytoskeleton in other cell lines are also independent of RNA and protein synthesis (24). We therefore conclude that βt2cAMP probably causes a rapidly reversible alteration in the PC12 cell cytoskeleton that results in neurite outgrowth.

The detailed mechanism by which βt2cAMP alters cytoskeleton organization is unknown, but it has been reported that a microtubule-associated protein (MAP), which may regulate microtubule assembly, is phosphorylated by a cAMP-dependent mechanism (19, 20). It appears that the transcription-dependent event in βNGF induction of neurite outgrowth involves the production of a gene product that regulates, in a more stable manner than βt2cAMP, the organization of microtubules and perhaps microfilaments.

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