

Selective Inhibition of Responses to Nerve Growth Factor and of Microtubule-associated Protein Phosphorylation by Activators of Adenylate Cyclase

Lloyd A. Greene, Steven A. Drexler, James L. Connolly,* Adriana Rukenstein, and Steven H. Green

Department of Pharmacology, New York University School of Medicine, New York 10016; and

*Department of Pathology, Harvard Medical School and Beth Israel Hospital, Boston, Massachusetts 02115.

Address correspondence to Dr. Greene.

Abstract. To study the influence of cAMP on cellular responses to nerve growth factor (NGF) and to use elevation of intracellular cAMP to probe the NGF mechanism, cultured PC12 pheochromocytoma cells were exposed to forskolin and cholera toxin. As in other cell types, the latter agents greatly increased PC12 cell cAMP levels. Such treatment also brought about a reversible, dose-dependent suppression of NGF-promoted regeneration of neurites. In support of the role of cAMP in this effect, regeneration blockage by forskolin was potentiated by phosphodiesterase inhibitors. When tested on NGF-stimulated initiation of process outgrowth, cholera toxin and forskolin exerted a dual effect. As in previous studies, these drugs, when applied along with NGF, significantly enhanced the initial formation of short cytoplasmic extensions. However, after ~3 d of NGF exposure, at which time such extensions begin to acquire the morphological and ultrastructural features of neurites, these agents suppressed process outgrowth. That is, the neurites were fewer in number, significantly less branched, and

much shorter than in control cultures. Such changes also occurred when these drugs were added to cultures that had been pretreated with NGF alone. Whereas forskolin and cholera toxin affect the formation and regeneration of neurites, these drugs did not interfere with the short-latency, transient changes in surface morphology that are triggered by NGF, nor did they inhibit transcription-dependent priming. In contrast, the rapidly occurring NGF-induced phosphorylation of tyrosine hydroxylase was suppressed. Moreover, forskolin and cholera toxin rapidly and selectively blocked the NGF-promoted phosphorylation of a set of microtubule-associated proteins known as chartins. Previous observations have suggested a causal relationship between NGF-induced chartin microtubule-associated protein phosphorylation and the formation and outgrowth of neurites. This is supported by the present data and provides a possible mechanism whereby elevated cAMP may interfere with neurite growth and regeneration.

THE nerve growth factor (NGF)¹ promotes a variety of responses in its target cells, including nerve outgrowth and regeneration, regulation of neurotransmitter synthesis, maintenance of survival, and stimulation of anabolic processes (for review cf. 11, 25, 36). The molecular mechanisms by which these actions are promoted remain largely unclear. One model system that has been extensively used to explore the NGF mechanism is the rat PC12 pheochromocytoma cell line (12, 13). PC12 cells undergo a number of well-defined responses to NGF and in its presence acquire a neuronal-like phenotype, including the capacity to generate and regenerate microtubule-containing neurites.

Possible mechanisms considered for the NGF mechanism have included mediation via activation of adenylate cyclase

and generation of elevated intracellular cAMP (cf. 35). However, although permeant analogues of cAMP can elicit some of the same responses in PC12 cells as does NGF, other NGF responses are not mimicked by these agents (cf. 13, 15 for review). In this respect, one area of some disagreement and confusion has been the effect of cAMP analogues or of cAMP-inducing agents on neurite outgrowth in PC12 cultures (cf. 17, 18, 21, 32, 35). It now appears to be generally accepted that cAMP analogues or inducing agents can stimulate PC12 cells to generate short (<50 μ m) extensions, but that these do not attain lengths or morphologies comparable to those of neurites elicited by exposure to NGF (9, 13, 17, 18, 32). Evidence has also been presented that permeant cAMP analogues may synergize the effects of NGF by enhancing the rate at which initial process formation and elongation occur in PC12 cultures (17, 18, 20, 33).

To further explore the influence of cAMP on the response

1. *Abbreviations used in this paper:* NGF, nerve growth factor; MAPs, microtubule-associated proteins.

of PC12 cells to NGF and to use elevation of intracellular cAMP as a tool to study the NGF mechanism, we have exposed cultures to agents, namely forskolin (33, 34) and cholera toxin (cf. 22), that selectively activate adenylate cyclase. We report that these agents can inhibit some responses of PC12 cells to NGF while leaving others intact. There is a dual response with respect to morphological events in that these agents lead to apparent enhancement of initial NGF-promoted process formation, but to suppression of the formation and regeneration of mature neurites. Forskolin and cholera toxin are also found to selectively inhibit the NGF-promoted phosphorylation of a set of microtubule-associated proteins (MAPs) designated (27) as chartins. Such data support a previously suggested (5) causal relationship between chartin MAP phosphorylation and NGF-induced neurite outgrowth.

Materials and Methods

Materials

Forskolin was purchased from Calbiochem-Behring Corp., La Jolla, CA, and was diluted from a 30-mM stock solution prepared in either dimethyl sulfoxide or ethanol. Cholera toxin was purchased from Schwarz/Mann, Spring Valley, NY.

Cell Culture

PC12 cells were cultured as previously described (12, 13) on collagen-coated tissue culture dishes. NGF was prepared as described by Mobley et al. (28) and, when present, was applied at a final concentration of 50 ng/ml. For experiments involving scanning electron microscopy, cells were grown on polylysine-coated coverslips as described elsewhere (7). Regeneration experiments were carried out and scored according to the protocols given by Greene (10).

Phosphorylation and Gel Electrophoresis

Cultures were labeled with [³²P]orthophosphate as previously described (16). Preparation of labeled microtubule and MAP fractions was as given by Burstein et al. (5) as were the methods used for SDS polyacrylamide gel analysis of labeled phosphoproteins.

Additional Methods

Levels of intracellular cAMP were determined with the use of protocols and kit (catalog No. NEX-132) purchased from New England Nuclear, Boston, MA; scanning electron microscopy was performed according to the methods given by Connolly et al. (6, 7). In these experiments, cells were fixed at 0.5, 1, 3, 7, 15, 45, and 120 min after addition of either forskolin or cholera toxin or, following pretreatment with forskolin or cholera toxin, after addition of NGF.

Binding and uptake of ¹²⁵I-NGF were carried out as described by Bernd and Greene (1). Determination of fast and slow binding components (23) was performed by removing the medium containing ¹²⁵I-NGF and adding 1 ml of medium containing a 500-fold excess (1 μg/ml) of unlabeled NGF. After a 30-min incubation on ice, this was removed and the dissociated ¹²⁵I-NGF measured in a gamma counter. The cells were further processed to distinguish surface-bound and internalized ¹²⁵I-NGF as described by Bernd and Greene (1).

Results

Effects of Forskolin and Cholera Toxin on PC12 Cell cAMP Levels

Fig. 1 shows the dose-response relationship between the concentration of forskolin added to the culture medium and PC12 cell intracellular cAMP levels. Basal levels were ~0.03

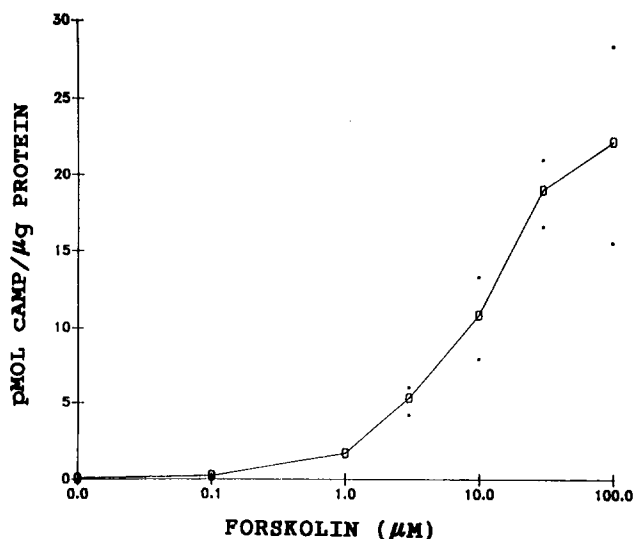


Figure 1. Effects of various concentrations of forskolin on the levels of cAMP in PC12 cells. PC12 cell cultures were exposed for 1 h to the indicated concentrations of forskolin and then analyzed as described in Materials and Methods for their specific intracellular contents of cAMP. The range of values obtained in duplicate assays is indicated by the dots.

pmol/μg protein and were increased by nearly 800-fold after 1 h of exposure to 100 μM forskolin. These findings are consistent with prior reports (31, 34). Cholera toxin, another activator of adenylate cyclase (22), also markedly raised PC12 cell intracellular cAMP. When applied at 10 μg/ml for 1 h, the cAMP levels rose by ~500-fold.

Effects on Neurite Regeneration

PC12 cells that have been pretreated with NGF for at least several days and then mechanically deprived of their processes exhibit NGF-dependent neurite regeneration within 24 h (reference 10 and Figs. 2 and 3). Fig. 3 shows the proportion of such cells regenerating neurites within 24 h after replating in the presence of NGF and various concentrations of forskolin. The latter agent suppressed neurite regeneration with a half maximal effect at ~4 μM. That this action was due to elevation of cAMP levels is supported by the observation that the phosphodiesterase inhibitors theophylline and isobutylmethylxanthine potentiated the inhibitory actions of forskolin on neurite regeneration and resulted in a shift in its half-maximally effective concentration to ~1.6 μM (Fig. 3). Also consistent with this view was the observation that cholera toxin suppressed neurite regeneration. At toxin concentrations of 1–10 μg/ml, regeneration was inhibited by ~75–80%.

At concentrations of forskolin or cholera toxin that blocked neurite regeneration, there were no visible indications of adverse effects on cell viability or attachment to substrate. Furthermore, as the data in Table I and Fig. 2 show, the inhibitory effects of forskolin on regeneration were reversed within 24 h after the drug was withdrawn from the culture medium.

The above findings show quantitative effects of forskolin and cholera toxin on NGF-promoted neurite regeneration. These agents also caused qualitative changes in the morphol-

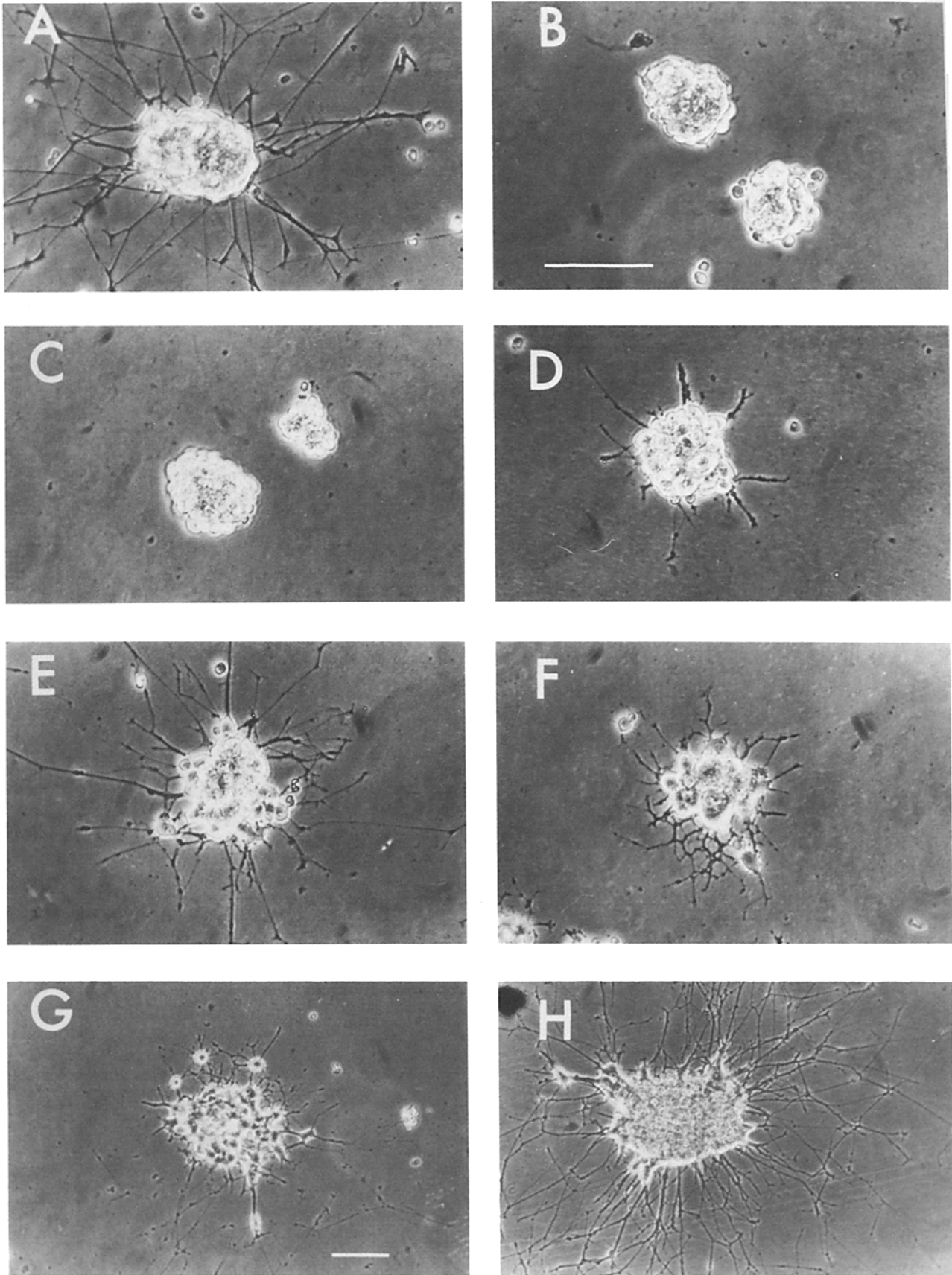


Figure 2. Effects of forskolin on neurite regeneration by PC12 cells. PC12 cells were pretreated with NGF for 2 wk, mechanically divested of their neurites, and subcultured (*A*) for 1 d in the presence of NGF; (*B*) for 1 d in the absence of NGF; (*C*) for 1 d in the presence of NGF and 30 μ M forskolin; (*D*) for 1 d in the presence of NGF and 3 μ M forskolin; (*E*) for 1 d in the presence of NGF and 1 μ M forskolin; (*F*) for 3 d in the presence of NGF and 10 μ M forskolin; (*G*) for 7 d in the presence of NGF and 10 μ M forskolin; and (*H*) for 5 d in the presence of NGF and 10 μ M forskolin and for an additional 2 d in the presence of NGF alone. Micrographs are of living cells taken under phase optics. Bars, 50 μ m. Bar in *B* provides calibration for *A-F*; bar in *G* provides calibration for *G* and *H*.

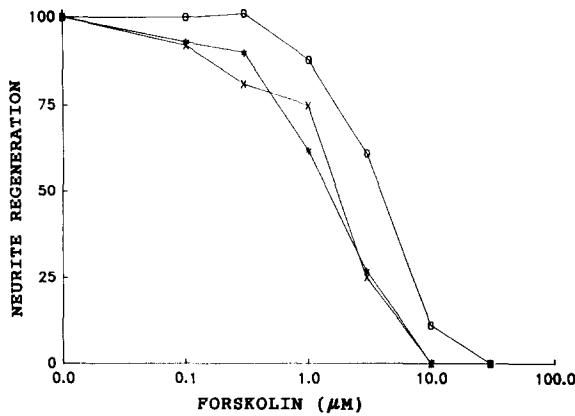


Figure 3. Effect of various concentrations of forskolin (with or without the presence of phosphodiesterase inhibitors) on the regeneration of neurites by PC12 cells. PC12 cells were pretreated with NGF for 11 d, divested of their neurites by mechanical means, and then subcultured as previously described (11) in the presence of NGF and the indicated concentrations of forskolin (0). In some cases, the medium was also supplemented with 10 mM isobutylmethylxanthine (*) or 10 mM theophylline (X). After 24 h, the cultures were scored for percentage of process-bearing cell clumps. At least 100 clumps were scored per condition. For each condition, the data were normalized so that 100% neurite regeneration equals the proportion of cell clumps regenerating neurites in the presence of NGF alone, less the number of cell clumps regenerating neurites in the absence of re-added NGF. For unsupplemented medium, these proportions were 97 and 12%, respectively; for medium supplemented with isobutylmethylxanthine they were 91 and 7%, respectively, and for medium supplemented with theophylline they were 93 and 11%, respectively. Comparable results were achieved in another, independent, experiment.

ogy of those neurites that grew out in their presence (see Fig. 2). These neurites tended to be shorter and possessed more varicosities than those produced in cultures containing NGF alone. The severity of these effects increased as the forskolin concentration was raised above 0.3 μM (Fig. 2). Such alterations in morphology were also present with concentrations of cholera toxin between 1 and 10 $\mu\text{g/ml}$ (data not shown).

Within several days after replating, additional neurites ap-

Table I. Reversibility of the Effect of Forskolin on Neurite Regeneration by Primed PC12 Cells

Day 1 after replating		Day 2 after replating	
Culture conditions	Neurite regeneration	Culture conditions	Neurite regeneration
	%		%
NGF	96	NGF	99
Control medium	9	Control medium	3
NGF + 30 μM forskolin	0	NGF + 30 μM forskolin	2
NGF + 30 μM forskolin	0	NGF	77
NGF + 10 μM forskolin	16	NGF + 10 μM forskolin	25
NGF + 10 μM forskolin	16	NGF	91

PC12 cells were pretreated with NGF for 2 wk and then passaged into new dishes in the presence of the indicated additives. After 1 d, the cultures were scored for percentage of cell clumps regenerating neurites. 100 clumps were scored per culture. The cultures were then washed 3 times with complete culture medium, re-fed with complete culture medium containing the indicated additives, and re-scored after an additional 24 h (day 2 after plating).

peared, even in the presence of 10–30 μM forskolin. However, these neurites were also stunted and beaded. With prolonged culture in the presence of 10–30 μM forskolin, the neurites retained their altered appearance for at least 10 d (see Fig. 2 G for an illustration at 7 d). Washout of the forskolin resulted not only in the outgrowth of greater numbers of neurites, but also in a return to the morphology present in control non-forskolin-treated cultures (Fig. 2 H).

Neurite Initiation

In contrast to regeneration, initiation of neurite outgrowth by PC12 cells without preexposure to NGF is relatively slow and occurs over a time course of several days (12). To test the effects of forskolin on neurite initiation, cultures were exposed to various concentrations of this drug (0.1–30 μM , in steps of threefold increments) in the presence or absence of NGF and observed at times of up to 3 wk. When administered without NGF, concentrations of forskolin from 1 to 30 μM elicited, within 24 h, the formation of short cytoplasmic spikes 3–20- μm long (Fig. 4 F). These extensions did not substantially elongate beyond this time. As previously described, NGF alone yielded a slow, progressive appearance of neurites that continued to elongate and branch throughout the entire period of treatment. When concentrations of forskolin above 1 μM were applied along with NGF, a dual response was observed (Figs. 4 and 5). For the first 3 d, the formation of short processes was more marked than that seen with either agent alone (Fig. 4). Beyond this time, the processes in the forskolin-treated cultures showed a dose-dependent decrease in their rate of elongation and extent of branching (Fig. 5). The neurites in these cultures took on the stunted form described above for cells regenerating neurites in presence of forskolin. This appearance persisted for as long as the cultures were maintained. Cotreatment with NGF and forskolin also led to an increase in the size of cell bodies greater than that seen with either agent alone (see Fig. 5). In addition to the effects that occurred when forskolin was applied to cultures at the same time as NGF, the inhibitory actions of this drug could also be observed in cultures that had been preexposed to NGF. When this drug was added to cultures that had been pretreated with NGF alone for 7 d, morphological changes occurred within several days so that the neurites took on an appearance similar to those in cultures with continuous exposure to NGF and forskolin (Fig. 5 F). The actions of forskolin on neurite initiation were also reversible in that withdrawal of this drug resulted, within a day, in commencement of process elongation, and, within several days, in the return of a more normal neurite morphology.

NGF Binding

One possible manner in which forskolin could affect responses of cells to NGF is via alteration of receptor binding. Two types of receptors for NGF have been described on PC12 cells and neurons (39). One class is of high affinity and appears to mediate both the biological actions of NGF as well as its internalization (1, 9). The dissociation of NGF from such receptors occurs relatively slowly and they have therefore also been termed slow NGF receptors (23). The second class of specific binding sites for NGF have an apparent affinity of $\sim 1/20$ that of the high affinity sites. These low

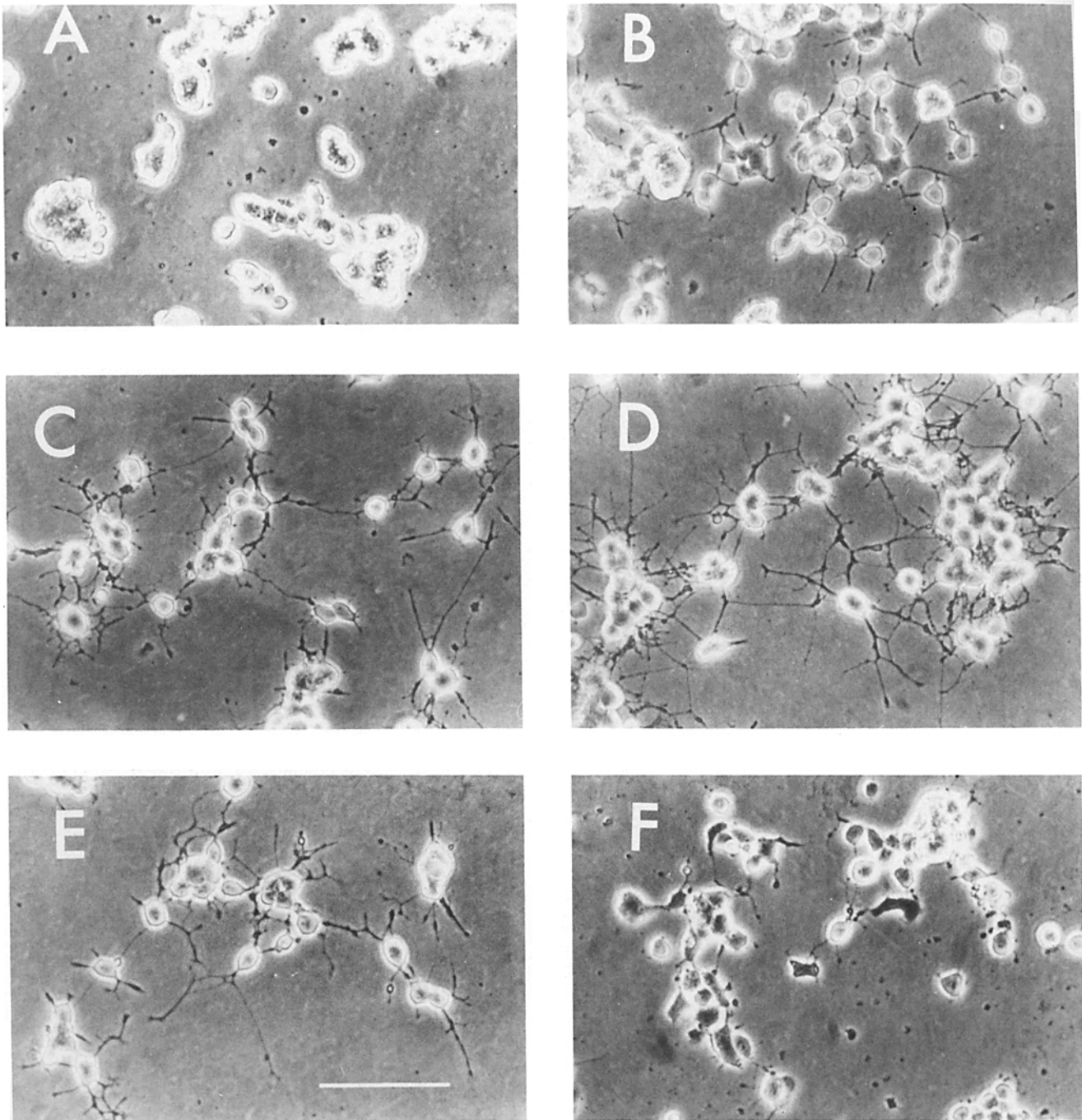


Figure 4. Effects of forskolin on the early morphological response of PC12 cells to NGF. Panels show micrographs (phase optics) of PC12 cells cultured for 3 d (*A*) in the absence of NGF; (*B*) in the presence of NGF; (*C*) in the presence of NGF and 30 μ M forskolin; (*D*) in the presence of NGF and 3 μ M forskolin; (*E*) in the presence of NGF and 0.3 μ M forskolin; and (*F*) in the presence of 30 μ M forskolin alone. Bar, 50 μ m.

affinity sites are also ~ 15 -fold more numerous, but do not appear to mediate NGF responses or uptake (9). Since NGF rapidly dissociates from these low affinity sites, they have also been termed fast NGF receptors (23). Experiments were performed (as described in Materials and Methods) to assess the effects of various concentrations of forskolin on binding of 125 I-NGF to both fast and slow NGF receptors and on the internalization of 125 I-NGF. The data in Table II indicate that concentrations of forskolin from 0.3 to 30 μ M caused

a modest apparent increase in the level of binding to slow receptors. At 30 μ M forskolin, there was also a decrease in the amount of NGF internalized. One possible interpretation of these data is that forskolin treatment somewhat slows the internalization of NGF-NGF/receptor complexes, thereby also enhancing surface levels of slow receptors. Overall, these data indicate that forskolin does not bring about a large change in the interaction of NGF with its receptors and that the above observed effects of forskolin on the response of

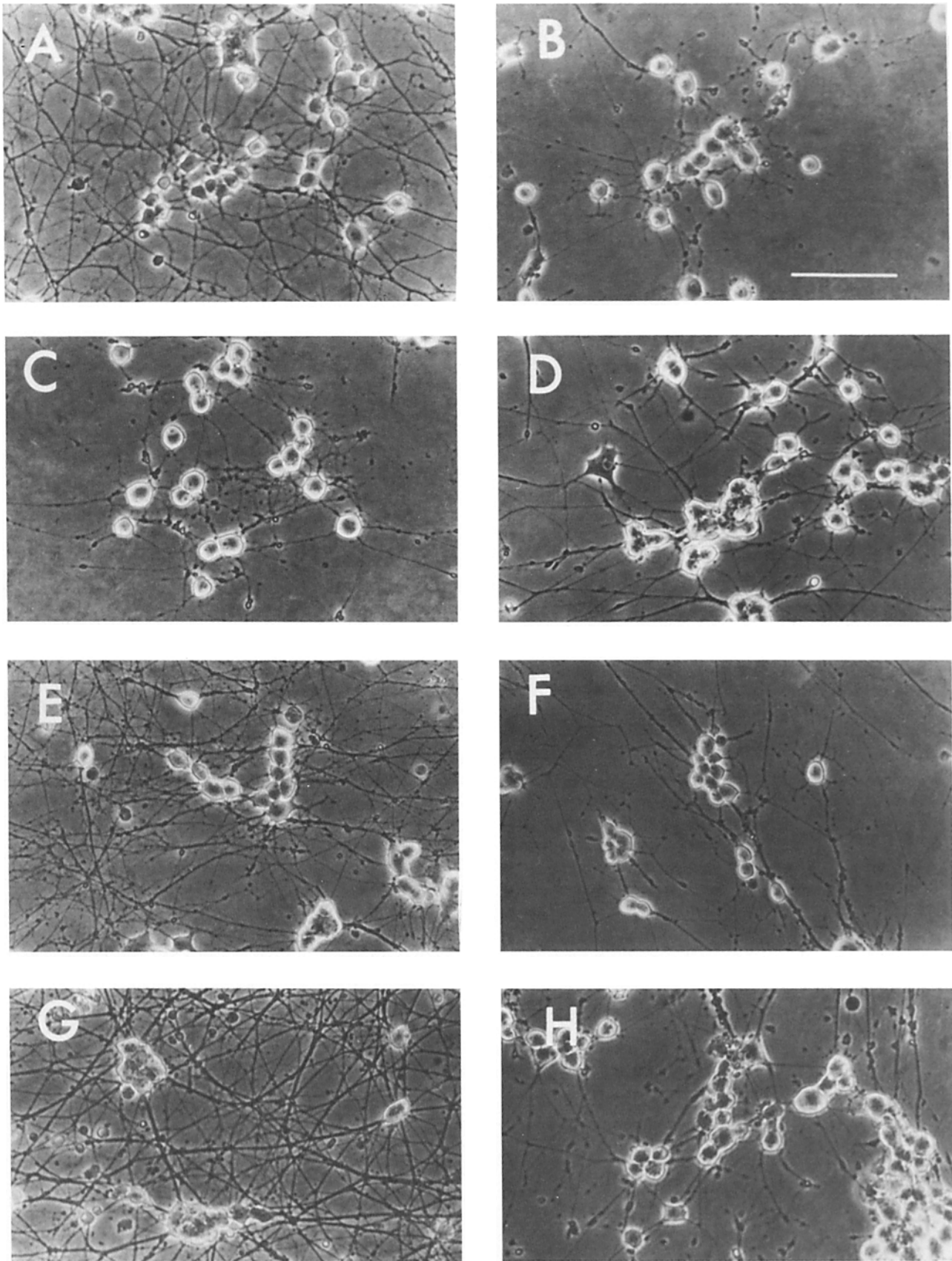


Figure 5. Long-term effects of forskolin on the morphological response of PC12 cells to NGF. Panels show micrographs (phase optics) of PC12 cells grown (A) for 7 d in the presence of NGF alone; (B) for 7 d in the presence of NGF and 30 μ M forskolin; (C) for 7 d in the presence of NGF and 10 μ M forskolin; (D) for 7 d in the presence of NGF and 3 μ M forskolin; (E) for 7 d in the presence of NGF and 0.3 μ M forskolin; (F) for 7 d in the presence of NGF alone and for an additional 3 d in the presence of NGF and 10 μ M forskolin; (G) for 17 d in the presence of NGF alone; and (H) for 17 d in the presence of NGF and 30 μ M forskolin. Bar, 50 μ m.

Table II. Effect of Forskolin on Binding and Uptake of ¹²⁵I-NGF

Incubation conditions	¹²⁵ I-NGF bound/10 ⁶ cells			¹²⁵ I-NGF internalized/10 ⁶ cells per h
	Total	Slow	Fast	
	<i>fmol</i>	<i>fmol</i>	<i>fmol</i>	<i>fmol</i>
Control medium	0.68 ± 0.07	0.25 ± 0.02	0.43 ± 0.04	1.26 ± 0.05
0.3 μM Forskolin	0.80 ± 0.14	0.31 ± 0.04	0.49 ± 0.06	1.35 ± 0.08
3 μM Forskolin	0.76 ± 0.16	0.32 ± 0.04	0.44 ± 0.07	1.11 ± 0.10
30 μM Forskolin	0.77 ± 0.12	0.36 ± 0.04	0.41 ± 0.05	0.69 ± 0.05

Cultures were preincubated with the indicated concentrations of forskolin for 30 min and then incubated in the presence of 28 pM ¹²⁵I-NGF and forskolin for 60 min. The binding conditions as well as means for distinguishing between surface and internalized NGF and between NGF bound to fast and slow receptors are as given in Materials and Methods. Each value is given as the mean ± SE (*n* = 3).

PC12 cells to NGF are unlikely to be due to interference with binding.

Priming

The phenomena of neurite initiation and regeneration by PC12 cells exhibit several differences. For instance, while the former is slow and requires RNA synthesis, the latter is rapid and can occur when RNA synthesis is blocked (4). These observations have led to the concept of NGF-dependent priming (4, 14). That is, NGF appears to activate a transcriptional pathway that influences PC12 cells to synthesize and store material that is required for neurite outgrowth. Pretreatment with NGF enables the cells, via this stored material, to rapidly regenerate neurites even in the presence of inhibitors of transcription. Thus, to test whether exposure to levels of forskolin that interfere with neurite growth and regeneration also affect priming, cultures were pretreated for 10 d with NGF either alone or in the presence of 30 μM forskolin and then assessed for regeneration under the various conditions described in Table III. As anticipated, cells pretreated with NGF alone underwent NGF-dependent neurite regeneration even in the presence of 5 μg/ml of actinomycin-D, but not when 30 μM forskolin was present. Moreover, when cells pretreated with both NGF and forskolin were replated in medium containing NGF but not forskolin, long normal-appearing neurites were regenerated within 24 h, irrespective of the presence of actinomycin-D. These findings indicate that forskolin does not interfere with the process of NGF-dependent priming.

Surface Morphology

Responses to NGF such as neurite regeneration and initiation and priming require from one to several days to take place. Experiments were therefore performed to test the effects of forskolin and cholera toxin on more rapid responses to the factor. Among the most rapidly occurring actions of NGF is the triggering of a stereotyped set of changes in cell surface morphology (7). Within several minutes of exposure to NGF, PC12 cells show extensive surface ruffling and the disappearance of microvilli. At ~15 min of treatment, the ruffles disappear. Exposure of the cells to 10 μg/ml of cholera toxin or up to 30 μM forskolin alone had no effect on surface morphology. In addition, pretreatment of PC12 cells with these drugs for 30–45 min had no effect on their subsequent surface responses to NGF (data not shown). These observations are consistent with prior findings that 1 mM dibutyryl cAMP does not alter PC12 cell surface architecture nor affect the changes in this parameter caused by NGF (6).

Rapid Changes in Protein Phosphorylation

Another rapid effect of NGF on PC12 cells is an alteration in the state of phosphorylation of several cell proteins (16, 19, 40). The most evident and consistently observable of such changes is the enhanced phosphorylation of a band that migrates with an apparent *M_r* of 60,000 by SDS PAGE (19). All or most of the responsive material in this band appears to be tyrosine hydroxylase (19, 24). Fig. 6 shows the patterns of phosphoproteins in PC12 cells that were exposed for 2 h to [³²P]orthophosphate in the presence of either control medium, 30 μM forskolin, NGF, or both 30 μM forskolin and NGF, and then resolved by SDS PAGE and detected by autoradiography. Forskolin itself caused several increases in labeling. The most reproducibly observed of these were bands at apparent *M_r*'s of 33,000, 41,000, and 121,000. Under these conditions, forskolin brought about little, if any, apparent change in phosphorylation of the *M_r* 60,000 band, while NGF, in agreement with previous reports (19, 24), brought about a significant increase in labeling of this material. As shown in Fig. 6, administration of forskolin strongly repressed this response to NGF. Identical results were obtained in 10 additional independent experiments with 30 μM

Table III. Forskolin Does Not Interfere with Priming of PC12 Cells

Pretreatment (10 d)	Replating treatment (24 h)	Cell clumps bearing neurites
		%
NGF	NGF	78
	Control medium	8
	NGF + forskolin	0
	Forskolin	0
	NGF + actinomycin	83
NGF + forskolin	NGF + forskolin + actinomycin	0
	NGF	82
	Control medium	3
	NGF + forskolin	4
	Forskolin	0
	NGF + actinomycin	92
	NGF + forskolin + actinomycin	1

PC12 cells were pretreated with NGF alone or with NGF + 30 μM forskolin for 10 d. The cells were then mechanically detached from the culture dishes and sheared of their neurites (if any) and washed extensively to remove forskolin and NGF. Aliquots of cells were then replated in fresh dishes in the indicated media and scored 1 d later for percentage of cell clumps bearing neurites. When present, the concentration of forskolin and actinomycin were 30 μM and 5 μg/ml, respectively.

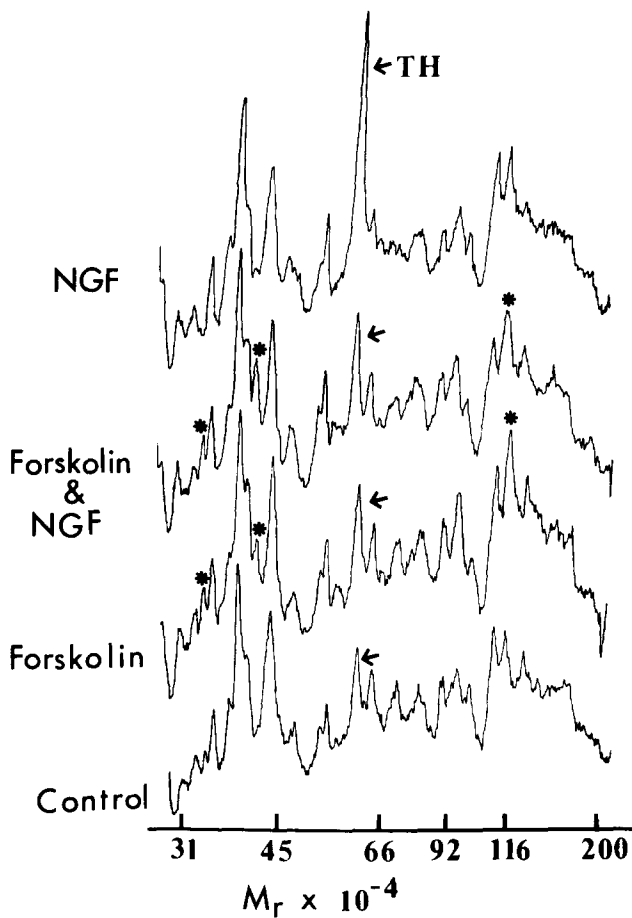


Figure 6. The effect of forskolin on the NGF-enhanced phosphorylation of tyrosine hydroxylase. PC12 cultures were labeled for 2 h with [³²P]phosphate in the presence of either no additive, 50 ng/ml NGF, 30 μ M forskolin, or 50 ng/ml NGF and 30 μ M forskolin. Cell proteins were then resolved by SDS PAGE (8–12% acrylamide gradient) and the labeled phosphoproteins were visualized by autoradiography. For each sample, equal numbers of TCA-precipitable cpm (50,000) were applied to the gel. The resulting autoradiogram was scanned on a densitometer (Hoefer Scientific Instruments, San Francisco, CA) and the resulting data are shown in the figure. The molecular mass calibration shown beneath the scans is based on the positions of molecular mass standards. Peaks denoted by TH and/or arrow indicate the position of tyrosine hydroxylase. Peaks marked with asterisks indicate bands whose relative densities were consistently affected by the presence of forskolin in this and other experiments.

forskolin and in one experiment with 3 and 10 μ g/ml of cholera toxin. In one experiment, suppression of the NGF response was also near-total with 10 μ M forskolin, substantial with 3 μ M forskolin, and partial with 1 μ M forskolin. Blockade was also achieved if the cells were prelabeled for 1.5 h with [³²P]phosphate and then exposed to NGF and 30 μ M forskolin for an additional 0.5 h. The latter finding appears to rule out the possibility that the observed effects were due to a rapid, slowly reversible forskolin-induced incorporation of unlabeled phosphate.

Long-term Changes in Phosphorylated MAPs

Exposure of PC12 cells to NGF for periods beyond 2–3 d brings about several characteristic changes in the levels of

several phosphoproteins. Among those readily observed by SDS PAGE and fluorographic analysis of PC12 cell proteins that have been labeled by exposure to [³²P]orthophosphate for 1–2 h are a species that appears to be phosphorylated β -tubulin (3, 5), a high molecular mass MAP (designated MAP1.2; 17) and a MAP of apparent M_r 64,000 (3, 5) designated 64-kD chartin MAP (see Fig. 7). At least in the

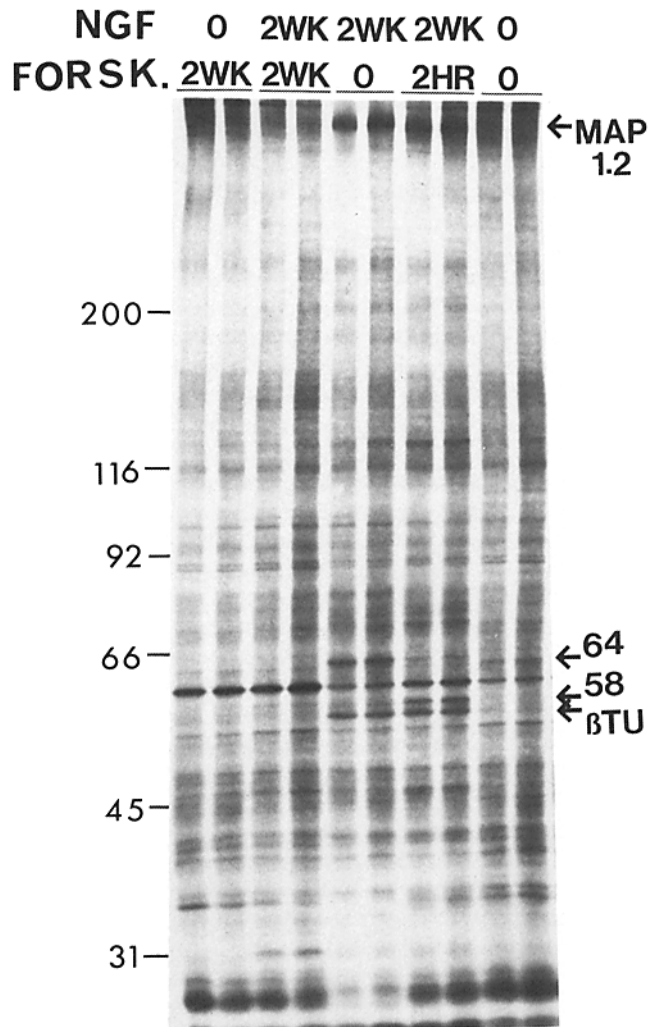


Figure 7. The effects of forskolin on phosphoproteins in NGF-treated PC12 cultures. PC12 cultures were labeled with [³²P]phosphate for 2 h after various pretreatments and in the presence of the various additives listed below. Cell proteins were then resolved by SDS PAGE (6–12% gradient) and the labeled phosphoproteins were visualized by autoradiography. For each sample, 100,000 cpm of TCA-precipitable material was applied to the gel. Treatments were as follows: 2 wk of exposure to, and labeling in the presence of 30 μ M forskolin (lanes 1 and 2 from the left); 2 wk of exposure to, and labeling in the presence of 30 μ M forskolin and NGF (lanes 3 and 4 from left); 2 wk exposure to NGF (lanes 5 and 6 from left); 2 wk of exposure to NGF followed by labeling in the presence of NGF and 30 μ M forskolin (lanes 7 and 8 from left); 2 wk in control medium (lanes 9 and 10 from left). Numbers on the left indicate the positions of molecular mass standards (given as $M_r \times 10^{-4}$). Arrows on the right indicate the positions of (from the top of the gels) MAP1.2, the M_r 64,000 phosphorylated chartin MAP, the M_r 58,000 forskolin-responsive band that is NGF-induced, and β -tubulin (β -TU).

latter case, the increase appears to be due to an NGF-dependent shift in degree of phosphorylation rather than to an increase in protein levels (3). Evidence has previously been presented that the 64-kD and other chartin MAPs are distinct from similarly sized tau MAPs (3, 27, 30) and that they may play a causal role in neurite outgrowth in PC12 cells and neurons (5, 29). Treatment with 1–30 μ M forskolin alone for up to 1 mo did not induce these MAP changes, nor did it substantially alter the overall phosphoprotein pattern (Fig. 7). Such long-term treatment did, however, result in several alterations, the most striking of which was enhancement of the levels of the phosphorylated M_r 60,000 band (Fig. 7). Although we have not verified the nature of this band, it most likely corresponds to tryosine hydroxylase. A time course study revealed that this effect on the M_r 60,000 band was evident within 16 h of forskolin exposure (data not shown).

In further experiments, PC12 cells were exposed for 2–4 wk to NGF and 10–30 μ M forskolin, and their phosphoproteins were then labeled and analyzed by SDS PAGE and autoradiography. In contrast to cells treated with NGF alone, those treated with NGF and forskolin did not show induction of the above-mentioned phosphoproteins (Fig. 7). Thus, levels of forskolin that adversely affect the extent and morphology of NGF-induced neurite outgrowth also suppress the expression of at least several NGF-regulated phosphorylated microtubule proteins.

Since the actions of forskolin and cholera toxin on neurite regeneration can be observed within a day, these agents were therefore also tested for their acute effects on protein phosphorylation in PC12 cell cultures that had been pretreated with NGF alone for at least 2 wk. As shown in Fig. 7, when such cultures were labeled with [32 P]phosphate in the presence of 30 μ M forskolin for 2 h, phosphorylation of the M_r 64,000 chartin MAP was substantially blocked. In contrast, under these conditions, the labeling of most cellular phosphoproteins, including β -tubulin, appeared to be unaffected. In some experiments, as in that shown in Fig. 7, MAP1.2 appeared to be slightly affected; in other experiments, there was no detectable effect on this species. The presence of forskolin also brought about a strong increase in the labeling of a band of apparent M_r 58,000. Overexposure of gels, such as that shown in Fig. 7, revealed that this band was slightly induced in response to long-term treatment with NGF alone. Findings similar to those described above were achieved either when treatment was with 5 μ g/ml of cholera toxin, when exposure to forskolin was for 20 h before labeling, or when cells were prelabeled with [32 P]phosphate for 1.5 h and then exposed to forskolin and [32 P]phosphate for an additional hour (data not shown).

In addition to enhancing phosphorylation of the M_r 64,000 chartin MAP, long-term exposure of PC12 cells to NGF also yields increased phosphorylation of additional chartin MAPs with apparent M_r 's of \sim 72,000 and 80,000 (3, 5). These chartins can be observed by analysis of solubilized microtubule fractions of PC12 cells (3, 5, 29). To obtain such fractions, cytoskeletal preparations are made by extraction of cell monolayers with a Triton-containing buffer in which microtubules are stabilized by inclusion of 6 M glycerol and exclusion of Ca^{++} ; the preparations are then treated with Ca^{++} in the cold to differentially disassemble and extract microtubule components (tubulin and MAPs). As in previ-

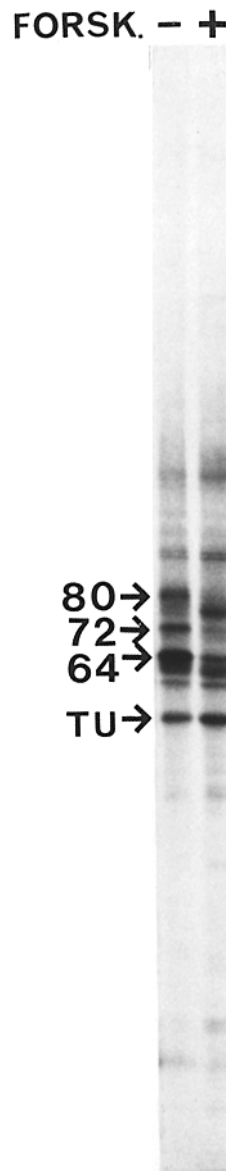


Figure 8. Effect of forskolin on phosphorylated microtubule-associated proteins in primed PC12 cells. PC12 cells were exposed to NGF for 17 d and then exposed to [32 P]phosphate for 2 h in the presence of either NGF (left lane) or NGF and 30 μ M forskolin (right lane). The cultures were then extracted as described in Materials and Methods to produce a fraction enriched in microtubule proteins and these were resolved by SDS PAGE (6–12% acrylamide gradient) and visualized by autoradiography. Equal numbers of TCA-precipitable cpm (25,000) were loaded in each lane. Numbers on the left indicate positions of the M_r 80,000, 72,000, and 64,000 phosphorylated chartins. TU indicates position of β -tubulin.

ous studies (3, 5), microtubule fractions prepared from primed PC12 cells that had been labeled with [32 P]phosphate contained three chartin MAP families and phosphorylated β -tubulin (Fig. 8). The identity of these components was confirmed by two-dimensional isoelectric focusing/SDS PAGE, under which conditions they exhibited their characteristic electrophoretic patterns (3, 29). Furthermore, as previously reported (3, 5), these species were greatly enhanced in level as compared with similar material from unprimed cells, and were selectively lost from the microtubule fraction when the cells were treated with anti-microtubule agents before extraction (data not shown).

The data in Fig. 8 show the effect of acute (2-h) exposure of primed PC12 cells to 30 μ M forskolin on phosphorylation of microtubule components. As was observed in whole-cell preparations, phosphorylation of the M_r 64,000 chartin MAP was greatly suppressed, while that of β -tubulin was largely unaffected. In addition, these data reveal that incorporation of phosphate into the M_r 72,000 and 80,000 chartin

MAPs was also significantly reduced in response to acute treatment with forskolin. Finally, the microtubule fraction from the forskolin-treated cells contained elevated levels of a component of apparent M_r 75,000.

Discussion

The data presented here indicate that agents causing large increases in the levels of intracellular cAMP in PC12 cells interfere with some responses to NGF while sparing others. The affected responses comprise those occurring over a variety of times after initial exposure to NGF and include enhanced phosphorylation of tyrosine hydroxylase (<2 h), neurite regeneration (<24 h), formation of long, branched neurites (>3 d), increases in the levels of phosphorylated MAPs (>3 d), and phosphorylation of the 64–80-kD chartin MAPs. The spared responses, likewise, also occur over a range of times and include membrane ruffling (<15 min), process initiation (<3 d), and priming (>3 d). One of the conclusions suggested by such observations is that the response of cells to NGF ultimately involves multiple pathways that differ from one another in their vulnerabilities to treatment with forskolin and cholera toxin.

There are several precedent studies regarding effects of cholera toxin or forskolin on responses of PC12 or chromaffin cells to NGF. Ziegler and Unsicker (41) noted that cholera toxin suppressed NGF-promoted process outgrowth from cultured neonatal rat chromaffin cells. Tischler and Slayton (38) reported that, in contrast, cholera toxin did not prevent either spontaneous or NGF-promoted process outgrowth from cultured adult human chromaffin cells. Ziegler and Unsicker also found enhanced process formation in PC12 cell cultures after 24 h of exposure to cholera toxin and NGF. Recently, Richter-Landsberg and Jastorff (32) reported an effect of forskolin similar to that described here in which early NGF-promoted neurite extension was enhanced, while, in the presence of 10–100 μ M forskolin for more than 6 d, neurite extension was inhibited. The latter authors also reported that at concentrations of 0.01–1 μ M, forskolin seemed to maintain and stabilize the NGF-promoted neurite network. In the present studies, while we did not systematically monitor the long-term effects of submicromolar forskolin concentrations, there was no dramatic evident potentiation of neurite outgrowth under such conditions.

Dual Effects on Process Outgrowth

Of particular interest is the observation of a dual effect of forskolin on process outgrowth. That is, the initial NGF-promoted outgrowth of processes is enhanced by the presence of forskolin, while, in contrast, the subsequent growth of long, branched neurites is suppressed. This phenomenon does not appear to be attributable to a delayed effect of the drug, since, when tested on neurite regeneration, its blocking actions were evident within 24 h. One possible explanation arises from considerations of the structural and compositional differences between those processes formed during initial exposure to NGF and the neurites that appear to form from these at later times or during regeneration. Ultrastructural studies indicate that the initial processes resemble cytoplasmic extensions and lack organized arrays of microtubule bundles (26). Only after at least 3 d of NGF treatment do the fibers acquire the parallel microtubular arrays that

typify neurites (26, 37). Concomitant with delayed formation of microtubule-containing neurites, there occurs an increase in microtubule stability (2), the proportion of total cellular tubulin that is polymerized (3, 8), along with specific increases in the levels of phosphorylated MAP1.2 (8, 16) and chartins (3, 5). The neurites that form during NGF-promoted regeneration appear to have the characteristics of established neurites rather than of initial cytoplasmic extensions. It thus appears that the short, initial extensions are not susceptible to the suppressive actions of forskolin, while neurites that contain microtubule arrays are susceptible to this agent. This, in turn, suggests that there may be mechanistic differences between the initial formation of short cytoplasmic extensions and the delayed establishment of neurites.

What might underlie the initial apparent synergistic effects of forskolin and cholera toxin on NGF-induced process formation? As confirmed here, agents that elevate or mimic cAMP in PC12 cells can themselves promote the formation of short cytoplasmic extensions (15, 17, 18, 21, 22) and appear to enhance the initial morphological actions of NGF (20, 21, 22, 32). Evidence has been presented that permeant cAMP analogues may promote such actions by leading to an enhanced stability of PC12 cell microtubules (20). Since the mechanism whereby NGF promotes microtubule polymerization and stabilization appears only after a delayed onset, elevation of cAMP levels during initial exposure to NGF may thus provide a more immediate and alternate effect on microtubules, thereby promoting initial process formation.

How might elevated cAMP suppress the regeneration and elongation of established neurites? One possibility is that elevated cAMP, either aside from, or due to, its limited actions on process formation, interferes with the delayed-onset mechanism by which NGF regulates microtubule polymerization and stability. One suggestive observation in this regard is the rapid and apparently selective effect of forskolin or cholera toxin on the NGF-induced phosphorylation of the 64–80-kD chartin MAPs. Chartins comprise one relatively abundant group of cellular proteins that selectively associate with microtubules (3, 5, 27, 29, 30). A variety of criteria have established that chartin MAPs are distinct from another set of MAPs, tau, of similar apparent M_r (3, 5, 27, 30). Exposure of PC12 cells to NGF promotes a slowly occurring, but substantial increase in the cellular level of highly phosphorylated chartin MAPs (3, 5). This effect is due to a shift in the degree of phosphorylation of chartins rather than to an increase in their total levels relative to other cell proteins (3). Recent experiments using lithium ion suggest a potential causal relationship between NGF-induced chartin MAP phosphorylation and NGF-promoted neurite outgrowth (5). That is, exposure of PC12 cells or sympathetic neurons to Li^+ was found to reversibly suppress the NGF-dependent outgrowth and regeneration of neurites. Under these conditions, Li^+ did not block NGF-promoted priming or several other responses to the factor. However, examination of protein phosphorylation in PC12 cells revealed that Li^+ rapidly and selectively blocks phosphorylation of the 64–80-kD chartin MAPs. Given the apparent role of MAPs in the formation of microtubules and of microtubules in the growth of neurites, such findings led to the hypothesis that NGF-induced chartin MAP phosphorylation may be a required element in microtubule assembly and neurite outgrowth (see also reference 29). The present experiments reveal an addi-

tional condition, elevation of intracellular cAMP, that interferes with neurite regeneration and growth and that rapidly and selectively suppresses NGF-induced phosphorylation of the 64–80-kD chitin MAPs. Such findings thus support the above hypothesis and indicate a likely mechanism whereby elevated cAMP may inhibit neurites, namely interference with NGF-dependent chitin MAP phosphorylation.

Mechanistic Considerations

It is clear that it is presently premature to attempt to draw conclusions about the detailed molecular mechanisms of the presently observed phenomena. The data do suggest that the various actions of forskolin and cholera toxin described here are mediated via activation of adenylate cyclase and the consequent elevation of intracellular cAMP. The only presently known shared effect of the two agents is, via different mechanisms, the activation of adenylate cyclase. Further support for this is provided by our observation that inhibition of NGF-promoted neurite outgrowth by forskolin is potentiated in the presence of phosphodiesterase inhibitors. There are several different means by which elevated cAMP could lead to alteration of responses to NGF. One is that the observed effects are mediated via activation of cAMP-dependent protein kinase (kinase A). A second is that cAMP, at the greatly increased levels generated in the presence of forskolin or cholera toxin, interacts with, and modifies the activity of a component (or components) other than protein kinase A. Ultimately, these, or other, mechanisms appear to lead rather specifically to inhibition of the pathways by which NGF leads to stimulation of the phosphorylation of tyrosine hydroxylase and of the chitin MAPs. Although a detailed picture of the events occurring between generation of cAMP and selective inhibition of responses to NGF is currently lacking, it is nevertheless evident that forskolin and cholera toxin represent valuable probes for dissecting the mechanism of action of NGF. One important example of this is the correlative finding that these agents block both NGF-dependent neurite outgrowth and chitin MAP phosphorylation.

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References

1. Bernd, P., and L. A. Greene. 1984. Association of ¹²⁵I-nerve growth factor with PC12 pheochromocytoma cells: evidence for internalization via high affinity receptors only and for long-term regulation by nerve growth factor of both high- and low-affinity receptors. *J. Biol. Chem.* 259:15509–15516.
2. Black, M. M., and L. A. Greene. 1982. Changes in colchicine susceptibility of microtubules associated with neurite outgrowth: studies with nerve growth factor-responsive PC12 pheochromocytoma cells. *J. Cell Biol.* 95:379–386.
3. Black, M. M., J. M. Aletta, and L. A. Greene. 1986. Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. *J. Cell Biol.* 103:545–557.
4. Burstein, D. E., and L. A. Greene. 1978. Evidence for both RNA-synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 75:6059–6063.
5. Burstein, D. E., P. J. Seeley, and L. A. Greene. 1985. Lithium ion inhibits nerve growth factor-induced neurite outgrowth and phosphorylation of nerve growth factor-modulated microtubule-associated proteins. *J. Cell Biol.* 101:862–870.

6. Connolly, J. L., S. A. Green, and L. A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. *J. Cell Biol.* 98:457–465.
7. Connolly, J. L., L. A. Greene, R. R. Viscarello, and W. D. Riley. 1979. Rapid, sequential changes in surface morphology of PC12 pheochromocytoma cells in response to nerve growth factor. *J. Cell Biol.* 82:820–827.
8. Drubin, D. G., S. C. Feinstein, E. M. Shooter, and M. W. Kirschner. 1985. Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule-assembly and assembly-promoting factors. *J. Cell Biol.* 101:1799–1807.
9. Green, S., R. E. Rydel, J. L. Connolly, and L. A. Greene. 1986. PC12 mutants possessing low- but not high-affinity NGF receptors neither respond to, nor internalize NGF. *J. Cell Biol.* 102:830–843.
10. Greene, L. A. 1977. A quantitative bioassay for nerve growth factor (NGF) activity employing a clonal pheochromocytoma cell line. *Brain Res.* 133:350–353.
11. Greene, L. A., and E. M. Shooter. 1980. The nerve growth factor: biochemistry, synthesis, and mechanism of action. *Annu. Rev. Neurosci.* 3:353–402.
12. Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 73:2424–2428.
13. Greene, L. A., and A. S. Tischler. 1982. PC12 pheochromocytoma cultures in neurobiological research. In *Advances in Cellular Neurobiology*. Vol. 3. S. Federoff and L. Hertz, editors. Academic Press, Inc., New York. 373–414.
14. Greene, L. A., D. E. Burstein, and M. M. Black. 1982. The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. *Dev. Biol.* 91:305–316.
15. Greene, L. A., D. E. Burstein, and M. M. Black. 1979. Cell culture studies on the mechanism of action of nerve growth factor. *Soc. Neurosci. Symp.* 4:153–171.
16. Greene, L. A., R. K. H. Liem, and M. L. Shelanski. 1983. Regulation of a high molecular weight microtubule-associated protein in PC12 cells by nerve growth factor. *J. Cell Biol.* 96:76–83.
17. Gunning, P. W., G. E. Landreth, M. Bothwell, and E. M. Shooter. 1981. Differential and synergistic actions of nerve growth factor and cyclic AMP in PC12 cells. *J. Cell Biol.* 89:240–245.
18. Gunning, P. W., P. C. Letourneau, G. E. Landreth, and E. M. Shooter. The action of nerve growth factor and dibutyryl adenosine cyclic 3',5'-monophosphate on rat pheochromocytoma reveals distinct stages in the mechanisms underlying neurite outgrowth. *J. Neurosci.* 1:1085–1095.
19. Halegoua, S., and J. Patrick. 1980. Nerve growth factor mediates phosphorylation of specific proteins. *Cell.* 22:571–581.
20. Heidemann, S. R., H. C. Joshi, A. Schechter, J. R. Fletcher, and M. Bothwell. 1985. Synergistic effects of cyclic AMP and nerve growth factor on neurite outgrowth and microtubule stability of PC12 cells. *J. Cell Biol.* 100:916–927.
21. Huffaker, T., T. Corcoran, and J. Wagner. 1984. Adenosine inhibits cell division and promotes neurite extension in PC12 cells. *J. Cell. Physiol.* 120:188–196.
22. Hynic, S., and G. W. G. Sharp. 1972. The effect of cholera toxin on intestinal adenylate cyclase. *Adv. Cyclic Nucleotide Res.* 1:163–174.
23. Landreth, G. E., and E. M. Shooter. 1980. Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. *Proc. Natl. Acad. Sci. USA.* 77:4751–4755.
24. Lee, K., P. J. Seeley, T. H. Muller, E. Helmer-Matyjek, E. Sabban, M. Goldstein, and L. A. Greene. 1985. Regulation of tyrosine hydroxylase phosphorylation in PC12 pheochromocytoma cells by elevated K⁺ and nerve growth factor: evidence for different mechanisms of action. *Mol. Pharmacol.* 28:220–228.
25. Levi-Montalcini, R. 1966. The nerve growth factor, its mode of action on sensory and sympathetic nerve cells. *Harvey Lect.* 60:217–259.
26. Luckenbill-Edds, L., C. Van Horn, and L. A. Greene. 1979. Fine structure of initial outgrowth of processes induced in a pheochromocytoma cell line (PC12) by nerve growth factor (NGF). *J. Neurocytol.* 8:493–511.
27. Magendantz, M., and F. Solomon. 1985. Analyzing the components of microtubules: antibodies against chitins, associated proteins from cultured cells. *Proc. Natl. Acad. Sci. USA.* 82:6581–6585.
28. Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 15:5543–5551.
29. Pallas, D., and F. Solomon. 1982. Cytoplasmic microtubule-associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. *Cell.* 30:407–414.
30. Peng, I., L. I. Binder, and M. M. Black. 1985. Cultured neurons contain a variety of microtubule-associated proteins. *Brain Res.* 361:200–211.
31. Rabe, C. S., S. J. Schneider, and R. McGee, Jr. 1982. Enhancement of depolarization-dependent neurosecretion from PC12 cells by forskolin-induced elevation of cyclic AMP. *J. Cyclic Nucleotide Res.* 8:371–384.
32. Richter-Landsberg, C., and B. Jastorff. 1986. The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. *J. Cell Biol.* 102:821–829.
33. Seamon, K. B., and J. W. Daly. 1981. Forskolin: A unique diterpene ac-

tivator of cyclic AMP-generating systems. *J. Nucleotide Res.* 10:201-224.

34. Seamon, K. B., and J. W. Daly. 1983. Forskolin, cyclic AMP and cellular physiology. *Trends Pharmacol. Sci.* 4:120-123.

35. Schubert, D., M. Lacorbiere, C. Whitlock, and W. Stallcup. 1978. Alterations in the surface properties of cells responsive to nerve growth factor. *Nature (Lond.)*. 273:718-723.

36. Thoenen, H., and Barde, Y.-A. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284-1335.

37. Tischler, A. S., and L. A. Greene. 1978. Morphological and cytochemical studies of a clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Lab. Invest.* 39:77-89.

38. Tischler, A. S., and V. W. Slayton. 1983. Cholera toxin does not prevent

neurite outgrowth from adult human chromaffin cells in culture. *Exp. Cell Res.* 143:454-456.

39. Yankner, B. A., and E. M. Shooter. 1979. Nerve growth factor in the nucleus: interaction with receptors on the nuclear membrane. *Proc. Natl. Acad. Sci. USA.* 76:1269-1273.

40. Yu, M. W., N. W. Tolson, and G. Guroff. 1980. Increased phosphorylation of specific nuclear proteins in superior cervical ganglia and PC12 cells in response to nerve growth factor. *J. Biol. Chem.* 255:10481-10492.

41. Ziegler, W., and K. Unsicker. 1981. Differential effects of cyclic AMP and cholera toxin on nerve growth factor-induced neurite outgrowth from adrenal medullary chromaffin cells and pheochromocytoma cells. *Dev. Brain Res.* 1:622-627.