Differential Inhibition of Nerve Growth Factor and Epidermal Growth Factor Effects on the PC12 Pheochromocytoma Line

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ABSTRACT Tests have been made of the action of the methyltransferase inhibitors 5′-S-methyl adenosine, 5′-S-(2-methyl-propyl)-adenosine, and 3-deaza-adenosine ± L-homocysteine thiolactone, on nerve growth factor (NGF)-dependent events in the rat pheochromocytoma line PC12. Each of these agents inhibited NGF-dependent neurite outgrowth at concentrations of the order of millimolar. Slow initiation of neurite outgrowth over several days and more rapid regeneration of neurites (=1 d) were blocked, as was the priming mechanism necessary for genesis of neurites. The inhibitions were reversible in that PC12 cells maintained for several days in the presence of inhibitors grew neurites normally after washout of these agents. Other NGF-dependent responses of the PC12 line (i.e., induction of ornithine decarboxylase activity [over 4 h], enhancement of tyrosine hydroxylase phosphorylation [over 1 h], and rapid changes in cell surface morphology [30 s onward]) were inhibited by each of the agents. In contrast, corresponding epidermal growth factor-dependent responses in ornithine decarboxylase activity, phosphorylation, and cell surface morphology were not blocked, but instead either unaffected or enhanced, by the methylation inhibitors. These inhibitors did not act by blockade of binding of NGF to high- or low-affinity cell surface receptors, though they partially inhibited internalization of [125I]NGF. The inhibition of rapidly-induced NGF-dependent events and the differential inhibition of responses to NGF and epidermal growth factor imply that the methyltransferase inhibitors specifically block one of the first steps in the mechanistic pathway for NGF.

Nerve growth factor (NGF) is a chemically-defined protein that exerts a major influence on the development of the sensory and sympathetic nervous systems (1–3). Its primary actions on target cells, including maintenance of survival, induction of neurite growth, and alterations in the metabolism of neurotransmitters, may be reproduced in vitro, thereby rendering questions of neuronal development accessible to cell culture techniques. The rat pheochromocytoma line, PC12 (4), is a useful resource for studies of NGF since, unlike sympathetic and sensory neurons in primary culture, these cells do not require NGF for survival. For this reason, both initiation of neurite growth and neurite regeneration may be studied in PC12 cultures and this has enabled transcription-dependent and transcription-independent pathways of neurite outgrowth to be identified (5). Finally, the ability of PC12 cells to divide in the absence of NGF and their clonal nature allow supply of large quantities of homogenous material for biochemical studies. (The PC12 cell system is discussed in detail by Greene and Tischler [6]).

In attempting to describe the actions of hormones on their target cells, attention has been given to transduction of signals across the cell membrane as a result of ligand-receptor binding. Various forms of covalent modification of cellular components are candidates for such signaling mechanisms. In particular, adenosyl methionine-dependent methylation, in

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1Abbreviations used in this paper: DAA, 3-deaza-adenosine; EGF, epidermal growth factor; HCTL, L-homocysteine thiolactone; MTA, 5′-S-methyl adenosine; NGF, nerve growth factor; ODC, ornithine decarboxylase; SIBA, 5′-S-(2-methyl-propyl)-adenosine.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 · FEBRUARY 1984 · 417–426
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addition to its general role in cellular metabolism, has been invoked as a mediator of cellular responses such as chemotaxis (7–9) and exocytosis (10). Methylation of phospholipids has been identified as an early step associated with binding of a ligand to its cell surface receptor and one that may be obligatory for activation of cellular responses to that ligand, e.g., in activation of lymphocytes (11) and mast cells (12) or bradykinin stimulation of fibroblasts (13). Recently, Pfenninger and Johnson (14) have observed NGF-dependent changes in phospholipid methylation for neurons in culture. The potential usefulness of inhibitors of NGF action for resolution of mechanistic pathways for this protein has led us to examine the action of methylation inhibitors in the PC12 system.

We report in this paper that inhibitors of methylation (5’S-5-6methyl adenosine [MTA], 5’S-(2-methyl-2-propyl)adenosine, [SIBA], and 3-deaza-adenosine 1±–homocysteine thiolactone [DAA ± HCTL]) reversibly block all of the tested responses of the PC12 cell to NGF. These responses range in their period of onset from 30 s to several days. Although NGF responses are inhibited by the agents, comparable responses of the PC12 cell to epidermal growth factor (EGF) are not inhibited, but rather are either unchanged or enhanced, under the same conditions.

MATERIALS AND METHODS

Sources of Materials: DAA was obtained from the Southern Research Institute, Birmingham, AL. MTA, SIBA, 2-chloro-adenosine, and adenosine arabinoside were from the Sigma Chemical Co. N²(R-phenyl-isopropyl)adenosine was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). (Structures of reagents are given in Enouf et al.)

Dissolution of Inhibitors: SIBA stock solution was made up at 500 mM in dimethyl sulfoxide. DAA and MTA were dissolved at 10 mM in appropriate incubation media that had been briefly heated to ~70°C.

Cell Culture Methods: MC12 cells were cultured in RPMI 1640 medium using methods described by Seeley and Greene (16). Protocols for assessment of neurite initiation and regeneration were given previously (5).

Ornithine Decarboxylase Assays and Scanning Electron Microscopy: The procedure for assay of ornithine decarboxylase has been described (17). Cultures were prepared for scanning electron microscopy using methods described by Connolly et al. (18).

Phosphorylation of PC12 Cells: Cultures used for phosphorylation were plated at a density of 1–2 × 10⁶ cells per 35-mm diameter dish culture. Immediately prior to labeling, each culture was washed three times with 2-ml aliquots of HEPES-buffered Krebs-Ringer solution containing 0.1% (wt/vol) glucose (KRHK) and was then incubated for 1 h at 37°C in 1 ml KRHK containing 100 μCi [³²P]inorganic phosphate (New England Nuclear, Boston, MA; carrier free). NGF or EGF solution, or an equal volume of KRHK, were added and incubation continued for another hour. (Methylation inhibitors were added 0.5 h prior to NGF or EGF.) At the end of incubation, cultures were cooled on ice, immediately washed three times with 2.5 ml of ice-cold phosphate buffered saline containing 1 mM EDTA and 1 mM EGTA, and taken up in 150–200 μl of Laemmli (19) sample buffer enriched with 2.5 mM EDTA and 2.5 mM EGTA. Samples were incubated in a boiling-water bath for 5 min. Proteins were subsequently separated on 32 cm SDS polyacrylamide gels (10 or 15%) using the protocol of Laemmli (19). The gels were dried and then autoradiograms were recorded using Kodak X-Omat film. Quantification was carried out over an approximately linear range of film density using a Joyce–Loebl scanning densitometer.

Synthesis of [¹²⁵I]NGF and Binding Experiments: NGF was iodinated using the method of Sutter et al. (20). At least 95% of radioactivity was trichloracetic-acid-precipitable. The specific activity, determined by bioassay, was 460 cpm pg⁻¹. Binding was carried out on attached cells in complete growth medium as described by Bernd and Greene (21). Distinction between binding of [¹²⁵I]NGF to high- and low-affinity (or respectively, "slow" and "fast") cell surface receptors was made by criteria described previously by Landereth and Shooter (22) and by Schechter and Bothwell (23). Cultures were labeled in complete medium with [¹²⁵I]NGF (1.5 ng ml⁻¹ [=50 pM]) for 35 min at 37°C, washed rapidly six times with ~2.5 ml aliquots of cold phosphate-buffered saline and then incubated on ice for 35 min in the presence of excess (2 μg ml⁻¹) unlabeled NGF. [¹²⁵I]NGF present in the medium at the end of this "cold chase" incubation period was considered to be bound to low-affinity ("fast") NGF receptors (22, 23), whereas that bound to the cell surface at the end of this period was classified as bound to high-affinity ("slow") NGF receptors (22, 23).

Surface-bound and internalized factor were distinguished by the differential release method of Haigler et al. (24). The efficacy of this technique for [¹²⁵I]NGF bound to PC12 cells was verified by autoradiography in conjunction with electron microscopy (25). S. P. Bernd and L. A. Greene. Counts associated with the high-affinity surface receptor were released by exposing the cultures to low-pH buffer (0.2 M acetic acid containing 0.5 M sodium chloride) (24, 25). [¹²⁵I]NGF that remained with the cells after removal of surface-bound material was recovered by scraping the cells into 0.1 M sodium hydroxide and was considered to be internalized NGF (24, 25).

For all experiments, control cultures were processed in parallel to determine "background" labeling with [¹²⁵I]NGF in the presence of excess (2 μg ml⁻¹) unlabeled material. Noncompetable ("background") counts lay in the range 5–20% of the total counts in any fraction. Any inhibitors were preincubated with cultures for 1 h at 37°C and were also present during incubation with [¹²⁵I]NGF.

Protein Determinations: Protein determinations were carried out as described in the Bio-Rad Manual, according to the method of Bradford (26).

RESULTS

Neurite Outgrowth

The primary morphological response of the PC12 to extended treatment with NGF is neurite growth of at least 1 mm long. This can be observed in two forms: (a) neurite outgrowth from cells that have received first exposure to NGF, which is relatively slow with respect to both initiation of growth and rate of elongation of neurites (≈30 μm d⁻¹); (b) rapid regeneration of neurites (≈250 μm d⁻¹) from so-called primed cells that have received several days of pretreatment with NGF and have then been mechanically divested of their original neurites in the course of transfer to new culture dishes (5).

We have investigated the effects of inhibitors of methylation on neurite regeneration by PC12 cells. Results are presented in Fig. 1 for regeneration over 1 d. MTA and SIBA inhibited neurite regeneration completely (Fig. 1, a and b). The IC₅₀ values were ≈1 mM for both inhibitors. Although cells in the presence of these agents failed to extend neurites, they were bright under phase-contrast optics and appeared otherwise healthy. They adhered less strongly to the substratum and were less polygonal and flattened than cells maintained with NGF in the absence of MTA or SIBA. Their appearance was therefore similar to that of "primed" cells replated in NGF-free medium. Results for inhibition of neurite regeneration by DAA are in Fig. 1 c. HCTL, which was a rather ineffective agent when used alone, potentiated DAA inhibition such that the IC₅₀ for DAA was ≈200 μM in the absence of HCTL and decreased to ≈5 μM if HCTL was simultaneously present at 1 mM. The increase in potency of DAA on addition of HCTL is consistent with the action of DAA as an inhibitor of methylation (27).

Two additional inhibitors of methylation, 2-chloro-adenosine and adenosine arabinoside also inhibited neurite regeneration (by 50 and 60% respectively at a concentration of 0.3 mM). Adenosine (≤10 mM) and N²(R-phenyl-isopropyl)-adenosine (an adenosine agonist, ≤1 mM), which are either inactive or very poor methylation inhibitors, did not affect neurite regeneration.

Is the initiation of neurite outgrowth, from PC12 cells not previously treated with NGF, also inhibited by these agents? Results for MTA are given in Fig. 2 a. After 7 d of NGF treatment, 85% of control cells (no MTA) had neurites,
Since it was possible that inhibitions of both neurite initiation and regeneration were artefacts of irreversible cell damage, experiments were carried out to test for reversibility of effects. Fig. 2 shows the results of such an experiment for MTA. Cultures of PC12 cells pretreated with MTA (3 mM) and NGF for 7 d and then washed free of inhibitor grew neurites in the same way as cultures that had received no pre-exposure to inhibitor. By 7 d of NGF, ~70% of cells had neurites, irrespective of the former presence or absence of inhibitor. Nor was there a significant difference in the rate of neurite outgrowth from the MTA-pretreated cultures as compared with controls. The MTA effects on neurite growth were thus completely reversible. Similar data were obtained for SIBA.

Figs. 3 and 4 illustrate studies for neurite regeneration. Cultures were “primed” by pretreatment with NGF for several days and then passaged in the presence of NGF and inhibitors (3 mM MTA, 2 mM SIBA, and 30 μM DAA + 1 mM HCTL). Neurite outgrowth, assessed one day later, was almost totally suppressed (Figs. 3 and 4). The inhibitors were then removed by washing and cultures were scored 1 and 2 d later. In each case, removal of inhibitor or inhibitors was followed by rapid regrowth of long neurites that were similar in number and length to those regenerated by cultures that had been maintained free of these agents (Figs. 3 and 4).

Fig. 2 also illustrates the inhibitory action of MTA on an additional aspect of the NGF response: priming (5). As noted above, pretreatment of PC12 cells with NGF (priming) gives them the capacity, via a transcription-dependent mechanism, to regrow neurites rapidly when passaged in the presence of NGF. Conversely, “non-primed” (naïve) PC12 cells generate neurites more slowly and there is a lag in neurite production. Cultures that had been treated for 7 d with NGF ± 3 mM MTA were replated in the presence of NGF but the absence of MTA. Those cells that had not been exposed to inhibitor regenerated neurites within 1 d whereas MTA-treated cells grew neurites at the low rate characteristic of PC12 cultures that had not been primed by NGF (Fig. 2, b–d). Since cultures that were pretreated with MTA alone grew neurites at the same rate as those that were never given inhibitor, it does not seem likely that failure to observe the regeneration response is due to the τreg for recovery from inhibitor being much greater than the τprim for loss of priming (5), but rather simply to inhibition by MTA of the process of priming itself. Similar experiments with SIBA indicated that this agent also inhibited priming of PC12 cells.

whereas the corresponding value was 1% for cultures containing 3 mM MTA. Similar levels of inhibition were achieved using 1 mM SIBA. Cells treated with these agents did not exhibit NGF-dependent flattening characteristic of control cells. Some cell death was caused by the methylation inhibitors; in particular, high concentrations of DAA and HCTL together were particularly cytotoxic over 2–3 d of treatment and hence the effect of the latter agents on neurite initiation could not be reliably assessed. For the experiments presented in Fig. 2, however, cell death caused by inhibitors never represented more than ~5% of the total number of cells. It is not likely therefore that a subpopulation of the cultures was being selected by these agents.

Since it was possible that inhibitions of both neurite initia-
Ornithine Decarboxylase (ODC) Activity

At what points in the mechanistic pathways activated by NGF do these inhibitory agents function? A number of biochemical and morphological responses of PC12 cells to NGF, of widely varying latency, have been examined for their susceptibility to the methylation inhibitors.

NGF increases the activity of ODC in PC12 by 20-40-fold. The period of maximum induction is ~4 h and the process is transcription-dependent (17, 28, 29). Induction of ODC by NGF is represented by the data in Fig. 5. There was a 37-fold increase in enzyme activity of PC12 cultures that had been incubated with NGF for 4 h. This induction was blocked by the same methylation inhibitors that prevented neurite outgrowth. For the experiment illustrated in Fig. 3, incubation with 2 mM MTA for the entire period of NGF treatment restricted the induction to sixfold; with 3 mM MTA, ODC levels were the same as those in control cultures. Hence MTA is an effective inhibitor of NGF-dependent induction of ODC. Dose-response experiments (not shown) revealed that MTA was active in this respect at the same concentrations as those that inhibited neurite outgrowth. This pattern of MTA inhibition of NGF-dependent ODC activity was reflected in data for SIBA given in Fig. 6. SIBA was an effective inhibitor at concentrations =1 mM.

The potential of agents such as MTA for nonspecific disruption of cellular metabolism may be approached indirectly, for the case of ODC activity, by examination of the EGF-dependence of ODC levels. EGF can also induce a transcription-dependent increase in ODC with a similar time-course to NGF (28, 29). Data in Fig. 5 for EGF-dependent induction of ODC were obtained from sister cultures of those used for the NGF incubations. The EGF-dependent induction of ODC was approximately eightfold, but MTA enhanced the EGF-dependent induction, rather than inhibited it, as was the case for NGF (Fig. 5); incubation with 2 or 3 mM MTA plus EGF gave stimulation of ODC levels of 36- and 25-fold respectively. (MTA alone caused no induction of ODC [Fig. 5].) The data in Fig. 5 represent a single experiment. There was some variability from experiment to experiment in the magnitudes of the EGF-dependent and EGF + MTA-dependent inductions of ODC. Usually MTA enhanced the EGF response and in no case was it inhibitory. Under the same conditions the NGF-dependent ODC induction was invariably blocked by MTA. Similar enhancements of the EGF response in ODC, with inhibition of the NGF-dependent induction, were obtained with SIBA at millimolar concentrations (Fig. 6). The converse effects of MTA or SIBA on ODC induction by NGF and by EGF are evidence against general disturbance of cellular metabolism by these agents as their means of inhibition of at least this short-term response of PC12 cells to NGF.
Protein Phosphorylation

Several groups have reported that NGF alters the state of phosphorylation of specific proteins for the PC12 line (30-34). A majority of the changes are rapidly induced (~10 min).

FIGURE 4 Reversibility of the effects of methylation inhibitors: neurite regeneration. PC12 cells were primed by exposure to NGF for 18 d and then passaged. Cultures were then incubated for 1 d with various inhibitors (as indicated) in the presence (striped bars) or absence (open bars) of 50 ng ml⁻¹ NGF. Control, inhibitor-free cultures were treated similarly. Neurite outgrowth was scored (processes > 100 μm) and cultures were then washed with medium to remove any inhibitors and incubated in NGF-containing medium for another 1 or 2 d. Neurite growth was scored at 1-d intervals. For the second and third days, stipled bars represent cultures deprived of NGF for the 1-d period immediately post passage (corresponding to open bars for day 1 after passage) and striped bars those cultures that received NGF on day 1 after passage (corresponding to striped bars for day 1).

FIGURE 5 The effect of MTA on induction of ODC by NGF and EGF. ODC activity was measured in PC12 cells that were harvested after treatment with NGF (50 ng ml⁻¹) or EGF (2 ng ml⁻¹) for 4 h (37°C). For MTA-treated cultures, the inhibitor was present throughout this period and also for the 0.5 h immediately preceding it (37°C). Error bars represent standard errors of mean values (n = 3).

FIGURE 6 The effect of SIBA on induction of ODC by NGF and EGF. Details as in the legend to Fig. 5.

post-translational modifications of cellular protein. We have tested the action of methylation inhibitors on rapid NGF- and EGF-dependent protein phosphorylation in PC12 cultures. As an example of the results, scans of portions of autoradiograms of SDS gels from phosphorylation experiments using 2 mM SIBA are given in Figs. 7 and 8. The strongest phosphorylation response to NGF was 60-kdaltons (Fig. 7). A significant fraction of radioactivity present in the 60-kdalton band has been found to be immunoprecipitated by antiserum specific for tyrosine monoxygenase (EC 1.14.16.2) (31, 35). 1-h incubation with NGF enhanced phosphorylation of the 60-kdalton protein about twofold and this enhancement was blocked by addition of 2 mM SIBA to the incubation medium (Fig. 7). There were smaller NGF-dependent enhancements of phosphorylation for bands corresponding to 54 and 65 kdaltons. These enhancements were also inhibited by 2 mM SIBA. (The extent of the inhibition was difficult to quantify accurately for these bands.) EGF also stimulated phosphorylation of the 54, 60, and 65-kdalton bands though to a lesser extent than NGF. SIBA action was also tested on EGF-dependent phosphorylations and in this case it stimulated EGF-dependent phosphorylation of the 60-kdalton band (Fig. 8). The pattern has thus repeated that for ODC activities: SIBA inhibited events contingent upon NGF but stimulated those contingent upon EGF.

Quantification of phosphorylation responses from several, separate experiments for the 60-kdalton band is presented in Table I. Inhibition of NGF-dependent phosphorylation occurred for MTA, SIBA, and DAA + HCTL at similar concentrations to those inhibiting neurite growth. DAA was a relatively ineffective inhibitor unless it was used in combination with HCTL (data not shown). By contrast, each agent or pair of agents was able to enhance EGF-dependent phosphorylation of the 60-kdalton band (Table I).

Scanning Electron Microscopy

Changes in cell surface morphology represent the most rapid responses of the PC12 cell to NGF that have been reported (18, 36). A stereotyped series of changes in cell surface architecture, which is closely defined in time, is initiated within 30 s of exposure to NGF. Essentially identical alterations in surface morphology occur in response to EGF (36). Observations were made by scanning electron micros-
copy of the effects of methylation inhibitors MTA and DAA ± HCTL on responses of PC12 cells to NGF or EGF. As in previous studies, untreated PC12 cells were ovoid in shape with numerous microvilli on their surfaces (Fig. 9a). Within 1 min of treatment of control cells (minus inhibitor) with NGF or EGF, ruffles appeared on the apical surface of the cell and, over the next 3 min, the ruffles increased in number whereas the microvilli simultaneously decreased in number. The extent of ruffling then lessened: by 5 min, ruffles were absent from the apical surface of the cell and by 15 min these structures were absent from the entire cell surface. Large blebs appeared at 45 min of treatment, became more numerous, and gradually disappeared over the following 3 h.

This sequence of responses to NGF was completely abolished by treatment of the cultures with MTA (3 mM). Substantial inhibition also occurred with DAA + HCTL (0.3 mM + 0.3 mM). DAA alone was less effective than when used in combination with HCTL. HCTL was quite ineffective as the sole inhibitor. In contrast, the sequence of alterations in cell surface form elicited by EGF was unaffected by the methyltransferase inhibitors MTA or DAA ± HCTL. The only change in cell morphology caused by the agents themselves was a moderate increase in length of microvilli. Fig. 9 is composed of representative cell morphologies for the various treatments and illustrates MTA inhibition of early NGF-induced ruffling of PC12 cells and the failure of such inhibition for EGF. In summary, the reactions to ligands observed by scanning electron microscopy fit into the pattern noted above: the effects of NGF, but not those of EGF, were blocked by methyltransferase inhibitors; for inhibition of NGF effects,


**TABLE I**

| Incubation condition | Ratio (plus factor/minus factor) |
|----------------------|---------------------------------|
| NGF                  | 1.6 ± 0.1                       |
| NGF + 2 mM SIBA      | 1.1 ± 0.1                       |
| NGF                  | 1.6 ± 0.1                       |
| NGF + 3 mM MTA       | 1.0 ± 0.1                       |
| NGF                  | 2.1 ± 0.1                       |
| NGF + 0.3 mM DAA + 0.3 mM HCTL | 0.99 ± 0.04 |
| EGF                  | 1.3 ± 0.1                       |
| EGF + 2 mM SIBA      | 1.6 ± 0.1                       |
| EGF                  | 1.7 ± 0.1                       |
| EGF + 3 mM MTA       | 1.9 ± 0.1                       |
| EGF                  | 1.3 ± 0.1                       |
| EGF + 0.5 mM DAA + 0.5 mM HCTL | 2.0 ± 0.1                       |

Incubation with [3H]inorganic phosphate and processing of cultures was carried out as described in Materials and Methods. NGF was present in the incubation medium at 50 ng ml⁻¹ and EGF at 30 ng ml⁻¹. Equal numbers of trichloroacetic acid precipitable counts for each of the conditions (control, NGF or EGF, inhibitor(s), inhibitor(s) plus NGF or EGF) were loaded onto the gels. Quantification was carried out by densitometric measurement of autoradiograms of SDS polyacrylamide gels. Results are expressed as the ratio of the integrated intensity of the 60-kdalton band in the presence of factor (NGF or EGF) to the corresponding intensity in the absence of factor (either with or without inhibitor). Uncertainties are standard deviations. The range of stimulation ratios for NGF and EGF were 1.5-2.1 (n = 4) and 1.3-1.7 (n = 3), respectively.

DAA and HCTL in combination were more potent than either agent used alone.

**Binding of [125I]NGF to PC 12**

There is significant evidence for initiation of NGF action on target cells by its binding to specific receptors on the outer surface (2, 3) as well as for internalization of the factor (1-3). Since all NGF responses of the PC12 cell that have been examined were blocked by inhibitors of methylation, it is possible that these agents interfere directly with NGF-receptor binding or with NGF internalization. There appear to be at least two types of NGF binding sites on the surfaces of PC12 cells and of other targets. These are of high- and low-affinity, are characterized also by different rates of dissociation of NGF from the NGF-receptor complex, and hence, are also known as slow and fast sites respectively (2, 3, 20, 24, 25). The two classes of surface receptor binding can be distinguished by their ability to release labeled NGF in the presence of excess unlabeled NGF at 4°C (22, 23). In addition, it has recently been shown that surface-bound NGF may be freed from the cell surface by exposure to buffer of relatively low pH (24, 25). As described in Materials and Methods and summarized in Table I, these methods were used to subdivide the various interactions of [125I]NGF with PC12 cells in the presence and absence of inhibitors. Except in the case of the DAA + HCTL, binding to low-affinity (fast) receptors was essentially unaffected by the methyltransferase inhibitors. Binding to high-affinity (slow) receptors was significantly increased by exposure to each of the inhibitors whereas internalization was decreased. Similar results were obtained in an experiment in which [125I]NGF was maintained at higher concentrations (5 ng ml⁻¹) and the time of exposure to NGF was increased (60 min).

**DISCUSSION**

A group of reagents, all adenosine analogs, and all inhibitors of S-adenosyl methionine-dependent methylations, blocked all of the tested responses of the PC12 line to NGF. Initiation of neurite growth, priming of neurite growth, and neurite regeneration were all inhibited by these agents. Rapidly-onsetting alterations in cell surface architecture caused by NGF, as well as slower, NGF-dependent enhancements of ODC activity and increases in the degree of phosphorylation of specific proteins, were inhibited. These phenomena covered a range of periods of treatment of PC12 cultures with NGF from tens of seconds (cell surface morphology) to several days (initiation of neurite growth). The events were transcription-dependent and -independent, biochemical, and morphological. The effective concentrations of inhibitors were approximately constant from one NGF response to the next. Action of inhibitors on neurite outgrowth from PC12 could be reversed, even after several days of treatment. Perturbation of binding and internalization of NGF by these agents was only partial. Additionally, for those responses of the PC12 cell that are common to both NGF and EGF, NGF responses were inhibited whereas those of EGF were either unaffected or enhanced. The effectiveness of the agents tested (MTA, SIBA, and DAA ± HCTL) against the most rapidly induced reactions of PC12 to NGF, the across-the-board character of the inhibitions and the differential action on NGF phenomena versus those of EGF indicate that these agents interfere specifically at a very early point in the mechanistic sequence for nerve growth factor action. But how do these inhibitors act on NGF and the PC12 cell? The lines of approach to this question are given below along with some preliminary experimental data. It is apparent that it would be premature to draw definite mechanistic conclusions at this time.

MTA, SIBA, and DAA ± HCTL are each inhibitors of methyl transferases that utilize S-adenosyl methionine (15). Data from experiments in which HCTL potentiated the inhibition of NGF-dependent events by DAA support the hypothesis that the present observations involve blockade of methyl group transfer (27). Each of the main classes of methyl transfer (i.e., to small molecules, nucleic acids, proteins, and lipids) may be inhibited either in vitro or in the intact cell by the reagents used in this study (27). The high concentrations (millimolar) that were required to block NGF-dependent events lie in the range of $K_i$ values for inhibition of methyl transfer to protein (15), and are, in general, in great excess of levels reported to be necessary for blockade of phospholipid methylation (e.g., reference 37). This contention is supported by preliminary experimental data which indicate that methylation of phospholipids in the PC12 cell is blocked by concentrations of inhibitors that are one to two orders of magnitude less than those required for blockade of NGF-dependent processes such as neurite growth (G. Ferrari and L. A. Greene, unpublished observations). For this reason, these data on NGF-dependent events in PC12 cultures do not appear to fit in place in a simple manner with involvement of phosphatidylethanolamine methylation in events at the cell surface such as have been reported for other systems (11, 12). NGF-stimulated phospholipid methylation has been observed in cultured peripheral neurons (14). It is not at present possible,
however, to relate these observations on neurons to the phenomena reported here for PC12 cells.

For the PC12 system, we have measured incorporation of $[^{3}H]CH_3$-groups from tritiated methionine into trichloroacetic acid-precipitable material in the presence of the protein synthesis inhibitor emetine (Seeley and Greene, unpublished observations). All of the reagents blocked the methylation of trichloroacetic acid-precipitable material. For example, $39 \pm 3\%$ of incorporation was blocked by $30 \mu M$ MTA and $74 \pm 1\%$ by $3 mM$ MTA. For DAA, $18 \pm 3\%$ of incorporation was blocked by $10 \mu M$ and $37 \pm 3\%$ by $300 \mu M$ DAA. In the presence of HCTL the inhibition of methylation was increased: $55 \pm 2\%$ for $10 \mu M$ DAA + $500 \mu M$ HCTL and $65 \pm 2\%$ for $300 \mu M$ DAA + $500 \mu M$ HCTL, respectively. (Values are given ± standard error in the mean; $n = 3$). One-dimensional gel electrophoretic separation of PC12 cell proteins, $[^{3}H]$methylylated in the presence of emetine as a protein synthesis inhibitor, have thus far failed to indicate NGF-dependent methylation of specific proteins (R. E. Rydel, P. J. Seeley, and L. A. Greene, unpublished observations). Electrophoresis was, in this case, carried out under the conditions of Laemmli (19) and not under those designed to protect protein carboxymethyl groups from degradation (e.g., reference 38).

If such results applied to the entire complement of cell proteins, or other cellular components such as lipids or nucleic acids, any methylation that mediated the effects of NGF would have to be ongoing, rather than switched by the factor.

An alternative to this mode of action as methylation inhibitors is that MTA, SIBA, and DAA behave as analogs of adenosine. Two principal loci for adenosine activity on the cell have been described. The first is the adenosine receptor and the second is the intracellular (P) site for adenosine inhibition of adenylyl cyclase (39). There are adenosine receptors on the PC12 cell and they are apparently stimulatory for adenylyl cyclase (type $A_2$) (40). There is no evidence, however, for capability of MTA, SIBA and DAA as adenosine agonists. Bruns (41) has determined that MTA is a noncompetitive inhibitor for the $A_2$ receptor from human fibroblasts, but the $K_I$ is $\approx 8 \mu M$. SIBA and DAA were found to be relatively inactive against the $A_2$ receptor with possible agonist and antagonist activities respectively at high concentrations (approximately millimolar (41]). Also, neither adenosine nor $N^\alpha$-(R-phenyl-isopropyl)-adenosine, a potent adenosine agonist (40), blocked NGF-dependent neurite regeneration in PC12 cultures. It is not possible therefore to set up a coherent hypothesis for inhibitory action on this basis. Little is known of P site influence on adenylyl cyclase in the cell (39). The $K_I$ for adenosine inhibition of adenylyl cyclase activity is in the range of $30 \mu M$ to $1 mM$, considerably higher than those required for receptor binding (approximately micromolar (39]).

MTA and SIBA have also been used to inhibit progesterone-induced meiosis in Xenopus laevis oocytes (42, 43). Inhibition in this case was accompanied by stimulation of adenylyl cyclase by SIBA, and this mode is thus a candidate for action of these materials in inhibiting NGF responses in PC12. Each of the methylation inhibitors increases cAMP levels in the PC12 cell by $\approx 2$-14-fold at levels of the agents that inhibit NGF responses (S. Drexler and L. A. Greene, unpublished observations). However, these modest increases in cAMP concentration do not appear to be linked in a simple fashion to inhibition of NGF-dependent responses since the potent adenylyl cyclase activator forskolin, which has previously been shown to stimulate adenylyl cyclase in the PC12 cell (44), does not cause inhibition of NGF responses at doses that increase cAMP to levels within this 2-14-fold range (S. Drexler, P. J. Seeley, and L. A. Greene, unpublished observations).

The experiments using $[^{125}]$NGF were carried out to test the possibility that the methyltransferase inhibitors worked directly on NGF-receptor binding or NGF internalization. With the exception of DAA + HCTL, the inhibitors did not significantly affect low-affinity binding, whereas NGF binding to high-affinity receptors was consistently enhanced. There is much evidence that the high-affinity (slow) receptors mediate some (if not all) NGF responses, including neurite outgrowth (3, 25). The binding data therefore point against inhibition of NGF responses by blockade of binding to the biologically significant receptor. Internalization of $[^{125}]$NGF, however, was significantly reduced by the inhibitors. Several points may be raised against this being the means of blockade of NGF action. First, loss of uptake was only partial; significant quantities of NGF were taken up by the cells in the presence of inhibitors. Second, one of the NGF responses studied (membrane ruffling) began before significant internalization took place. Third, evidence has been presented that NGF internalization is not required for neurite growth (45). Thus it does not appear that suppression of NGF-dependent events by methylation inhibitors is caused by loss of binding or internalization. It is not possible, however, to exclude involvement of a small subclass of receptors in the mode of inhibition.

In summary, MTA, SIBA, and DAA ± HCTL inhibit all tested PC12 responses to NGF by a pathway that is specific and reversible. Since these responses include ones activated within $30 s$ of exposure of the cell to NGF, it is likely that a very early mechanistic step is affected by these agents. It is possible that the inhibitors function by blockade of transmethylation. However, the reasons for the efficacy of these materials remain to be determined.
FIGURE 9  Effects of MTA on NGF- and EGF-induced changes in surface architecture of PC12 cells. PC12 cultures that had not been previously exposed to NGF or EGF were incubated with various combinations of reagents, fixed, and processed for scanning electron microscopy as described (18). The concentrations of NGF, EGF, and MTA in the culture medium were 50 ng ml⁻¹, 10 ng ml⁻¹, and 3 mM, respectively. (a) A PC12 cell that was not exposed to NGF, EGF, or MTA. Note the microvilli and small blebs on the surface of the cell and the absence of ruffles. (b) Two PC12 cells that were treated with NGF for 1 min. Note the large ruffles on the apical surface of the cells and decreased number of microvilli compared with the cell in a. (c) PC12 cell preincubated with MTA for 30 min and subsequently treated with NGF for 1 min. The ruffling response associated with NGF treatment is completely absent (compare b). The microvilli of this cell are more prominent than for the inhibitor-minus cell shown in a. (d) Two PC12 cells treated with EGF for 1 min. The ruffling response is similar to that for NGF (compare b). (e) Two PC12 cells preincubated with MTA for 30 min and subsequently treated with EGF for 1 min. MTA failed to block the ruffling response to EGF and the cells thus appear similar to those in d. Bars, 1 μm. × 5,200 (a, d, and e); × 6,000 (b and c).
### Experimental Details

Experimental details are given in Materials and Methods. Values each represent mean ± standard error (n = 3) femtomoles [3H]NGF per milligram cell protein.

We gratefully acknowledge the excellent technical assistance of Ms. Margaret DiPiazza.

This work was supported by grants from the National Institutes of Health (NS 16036 and AM 26920) and from the March-of-Dimes Birth Defects Foundation. L. A. G. is the recipient of a Career Development Award from the Irma T. Hirschl Foundation.

Received for publication 2 June 1983, and in revised form 24 October 1983.

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### Table II

**Effects of Inhibitors of Methylation on Binding and Internalization of [3H]NGF by PC12 Cells**

| Treatment | High-affinity (slow) | Low-affinity (fast) | Internalized | Total |
|-----------|----------------------|---------------------|--------------|-------|
| Control   | 1.7 ± 0.4            | 1.9 ± 0.5           | 8.6 ± 0.9    | 12.2 ± 1.8 |
| 3 mM MTA  | 3.4 ± 0.3            | 2.4 ± 0.1           | 5.2 ± 0.2    | 11.0 ± 0.6  |
| 2 mM SIBA | 2.8 ± 0.2            | 2.0 ± 0.3           | 3.5 ± 0.4    | 8.3 ± 0.9   |
| 0.3 mM DAA| 3.3 ± 0.2            | 1.1 ± 0.2           | 4.9 ± 0.2    | 9.3 ± 0.6   |
| + 0.3 mM HCTI |                    |                     |              |        |

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