

Nerve Growth Factor Treatment Enhances Nicotine-stimulated Dopamine Release and Increases in Cyclic Adenosine 3':5'-Monophosphate Levels in PC12 Cell Cultures¹

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Abstract

In order to examine the relationship between cyclic AMP (cAMP) levels and evoked neurotransmitter release, experiments have been performed with cultures of clonal rat PC12 pheochromocytoma cells. Stimulation of the release of endogenous dopamine by nicotine in these cultures is calcium-dependent and blocked by *d*-tubocurarine, a specific nicotinic cholinergic antagonist. Similarly, nicotine causes increases in cAMP levels in PC12 cell cultures that are calcium-dependent and blocked by *d*-tubocurarine. Cultures treated for 6 days or longer with 2×10^{-9} M nerve growth factor (NGF) release a 3- to 4-fold greater amount of dopamine than do control cultures in response to a maximal concentration of nicotine. Correspondingly, nicotine causes a 3-fold greater increase in cAMP levels in the NGF-treated cultures than in the controls. These results suggest that stimulation of the nicotinic cholinergic receptor in PC12 cells results in some manner in the activation of adenylate cyclase and further support the notion that cAMP is involved in the process of neurotransmitter release.

The results of several investigations employing a wide variety of *in vivo* and *in situ* preparations have suggested that cAMP may be involved in the process of neurotransmitter release. Most of this evidence has been indirect, i.e., cAMP analogues and phosphodiesterase inhibitors have been shown to enhance the release of transmitter from a variety of neuronal tissues (e.g., Wooten et al., 1973; Westfall et al., 1976; Standaert and Dretchen, 1979). However, none of these investigations has determined whether secretagogue-induced increases in nerve terminal cAMP levels are associated with evoked release.

Additionally, although Guidotti and Costa (1974) demonstrated that nicotinic stimulation of the adrenal gland results in an increase in adrenomedullary cAMP levels, the relationship of this increase to catecholamine release has been questioned. Jaanus and Rubin (1974) and Rubin (1975) concluded that the increase occurred too

long after stimulation of the adrenal to be involved in the regulation of release, while Serck-Hanssen (1974) and Boonyaviroj and Gutman (1977) suggested that the cAMP increase was actually elicited indirectly, by the action of released catecholamines on a chromaffin cell-surface β -adrenergic receptor coupled to adenylate cyclase. Further, the precise mechanism of this cAMP increase remains obscure. While nicotinic agonists cause the increase *in vivo* and an acceleration of cAMP formation in adrenal slices *in vitro*, these agents have no effect on the activity of adenylate cyclase in adrenal membrane preparations (Guidotti and Costa, 1974).

The lack of a clear relationship between cAMP levels and neurotransmitter release in previous studies may be due at least in part to the cellular heterogeneity of the systems employed. In order to examine the relationship between cAMP levels and release in a more defined system, we have performed experiments with cultures of clonal rat PC12 pheochromocytoma cells. This cell line, originally isolated by Greene and Tischler (1976) from a transplantable pheochromocytoma tumor, has been shown to be a useful model system for study of the synthesis (Greene and Rein, 1978), storage (Schubert and Klier, 1977; Roda et al., 1980), and release (Ritchie, 1979; Greene and Rein, 1977a, b) of catecholamines. Under routine culture conditions, PC12 cells resemble rat adrenal chromaffin cells, their nonneoplastic counterpart (Tischler and Greene, 1978). However, when cultured for extended periods in the presence of nanomolar levels of nerve growth factor (NGF), PC12 cells cease dividing, extend neurites, and acquire many of the characteristics of cultured sympathetic neurons (Greene and Tischler, 1982). The current series of experiments was undertaken in order to examine the relationship between nicotine-stimulated catecholamine release and increases in cellular cAMP levels in control and NGF-treated PC12 cell cultures.

Materials and Methods

Cell culture. Stock cultures of PC12 cells were grown in 85% Roswell Park Memorial Institute Medium 1640 (RPMI 1640), 10% (v/v) heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, with 50 units/ml of penicillin and 25 μ g/ml of streptomycin (Greene and Tischler, 1976) and were subcultured about once a week. For experiments, cells were plated on poly-L-lysine-coated tissue culture dishes (Yavin and Yavin, 1974) to which they adhere firmly. For NGF-treated cultures, cells were inoculated at an initial density of 4×10^4 /cm² on poly-L-lysine-coated dishes and cultured for 2 to 14 days in medium supplemented with 50 ng/ml β -NGF.

Measurement of catecholamine release. All of the experiments described were performed with the cultures maintained at 37°C in a controlled-temperature water bath. Serum-containing culture medium was removed and replaced with prewarmed oxygenated Krebs-Ringer-Henseleit (KRH) buffer (Greene and Rein, 1977a, b). After a 15-min incubation to allow the cultures to equilibrate, the buffer was removed and replaced with fresh KRH (to measure spontaneous release) or with KRH containing nicotinic agents (to measure evoked release). After 2 min of incubation, the release medium was removed and rapidly brought to pH 2 with 5 N HCl to ensure stability of the catecholamines. The cultures were treated with ice-cold 10% trichloroacetic acid (TCA) and scraped from the dish with a rubber policeman; 200 ng of

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dihydroxybenzylamine (DHBA) were added to the samples to assess recovery of catecholamines, and cellular proteins were removed by centrifugation. After purification by chromatography on alumina (Anton and Sayre, 1962), catecholamines from culture media and cell extracts (under routine culture conditions primarily dopamine (DA)) (Greene and Rein, 1978) were measured by high-pressure liquid chromatography (HPLC) and electrochemical detection. The amount of DA in the samples was estimated from the ratio of the DA peak height on the chromatogram to that of the internal standard, DHBA. Release is expressed as the percentage of cellular stores of DA released, i.e., the amount of DA in the medium divided by the sum of the amounts in the medium and in the cells at the end of the release incubation period multiplied by 100.

cAMP measurements. Cultures were initially treated as described for the measurement of catecholamine release. After the 2-min incubation period, the medium was rapidly brought to 10% in TCA by the addition of the appropriate amount of 50% TCA. After adding approximately 10,000 cpm of [3 H]cAMP to the samples to assess recovery of cAMP, proteins were removed by centrifugation. cAMP was purified from the acid supernatant by chromatography on Dowex 50-cation exchange resin by the method of Matsuzawa and Nirenberg (1975). Eluates from the Dowex columns were acetylated (Harper and Brooker, 1975), and cAMP was measured by a specific radioimmunoassay (Steiner et al., 1972). All cAMP measurements were corrected for the recovery of [3 H]cAMP, and cAMP levels are expressed as picomoles per milligram of protein, measured by the method of Bradford (1976) with bovine serum albumin as standard.

Materials. RPMI 1640 was obtained from Gibco (Grand Island, NY). Horse serum was from K.C. Biologicals (Lenexa, KA); fetal calf serum was from Sterile Systems (Logan, UT); HEPES (free acid), nicotine, poly-L-lysine, and Sephadex G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). AG50-WX4 cation exchange resin (Dowex 50) was from Bio-Rad Laboratories (Berkeley, CA). Alumina was from Woelm (Eschwege, West Germany) and was acid washed according to the procedure of Anton and Sayre (1962). All other reagents were from Baker Chemical Co. (Phillipsburg, NJ). Polystyrene tissue culture dishes were Falcon brand (Becton-Dickinson, Cockeysville, MD). [3 H]cAMP (38.2 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [125 I]cAMP tracer for the radioimmunoassay was prepared by iodination of 3'-succinyl cAMP tyrosine methyl ester (Sigma) and subsequent purification on Sephadex G-25. β -NGF was purified from male mouse salivary gland by the method of Mobley et al. (1976). The purified protein was stored at -20°C at a concentration of 1 mg/ml in 0.2% acetic acid; under these conditions, the preparation retained its full biological activity for more than 1 year. The HPLC system used was a Beckman-Altex (Berkeley, CA) with a Lichrosorb RP-18 $10\text{-}\mu\text{m}$ 250 \times 4.6 mm column (EM Reagents, Gibbstown, NJ) and an electrochemical detector from Bioanalytical Systems (West Lafayette, IN). Detector potential was set at 0.7 V positive to the reference electrode.

Results

Control PC12 cells contain 16.3 ± 1.8 nmol of DA/mg of protein, while those treated with NGF for 6 d or longer contain 4.4 ± 0.3 nmol of DA/mg of protein, values similar to those reported by Greene and Tischler (1976). These investigators further determined that if catecholamine levels are expressed as nanomoles of DA per 10^6 cells, those of control and NGF-treated cells are comparable, suggesting that NGF stimulates protein synthesis in PC12 cells, rather than causing a reduction in DA levels. Dopamine release in response to various concentrations of nicotine is illustrated in Figure 1; in both types of culture, this response is maximal at 10^{-4} M nicotine with a decrement at higher concentrations, possibly due to persistent depolarization-induced blockade (Greene and Rein, 1977b). Expressed as net dopamine release (spontaneous release subtracted), control cultures release 3% and NGF-treated cultures release 11 to 13% of their endogenous DA stores in response to 10^{-4} M nicotine. In both types of culture, release is about 80% calcium-dependent and blocked by the specific nicotinic cholinergic antagonist *d*-tubocurarine (10^{-5} M; Table I).

cAMP levels were measured in both types of culture after incubation for 2 min in KRH buffer containing various concentrations of nicotine (Fig. 2). Nicotine at 10^{-4} M causes a 95% and a 260% increase in cAMP levels in control and NGF-treated cultures, respectively; the increase in this response after long-term NGF treatment is similar to the increase in evoked DA release. Additionally, these

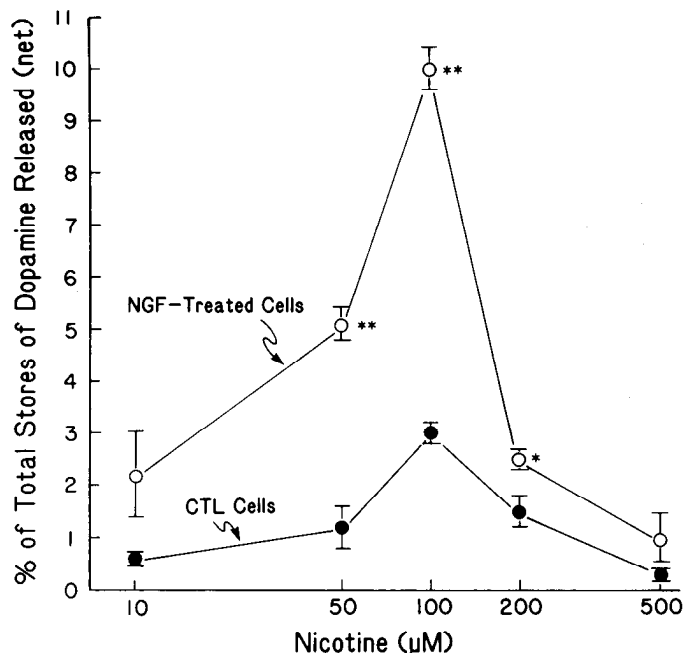


Figure 1. Dose-response curve for nicotine-stimulated DA release in control and NGF-treated PC12 cell cultures. Cultures were incubated for 2 min in KRH buffer containing the indicated concentrations of nicotine, and DA release was measured as described under "Materials and Methods." Release is expressed as net release (spontaneous release subtracted). Each point represents the mean \pm SE of at least three determinations on separate cultures. NGF-treated cultures were grown for 2 weeks in medium supplemented with 50 ng/ml of purified NGF. *, significantly different from control ($p < 0.05$); **, significantly different from control ($p < 0.01$).

TABLE I

Calcium dependence and curare sensitivity of nicotine-stimulated DA release and increases in cAMP levels

Control or NGF-treated PC12 cell cultures were incubated for 2 min in nicotine-containing KRH under the indicated conditions, and DA release and cellular cAMP levels were measured as described. NGF-treated cultures were grown for 2 weeks in medium supplemented with NGF.

	10^{-4} M Nicotine ^a (%)	10^{-4} M Nicotine 0 Ca^{++} ^b (%)	10^{-4} M Nicotine + 10^{-5} M <i>d</i> -Tubocurarine ^c (%)
Dopamine re- lease ^c			
Control cells	3.1 ± 0.2	0.7 ± 0.3	0.1 ± 0.4
NGF-treated cells	11.7 ± 0.4	2.2 ± 0.3	0.3 ± 0.6
cAMP increase ^d			
Control cells	1.96 ± 0.04	1.34 ± 0.1	1.06 ± 0.06
NGF-treated cells	3.53 ± 0.09	1.57 ± 0.13	0.99 ± 0.1

^a 2.6 mM calcium.

^b 0 Ca^{++} medium contained 1 mM EGTA.

^c DA release is expressed as net percentage of stores of DA released.

^d cAMP increase is expressed as the ratio of nicotine-stimulated to basal levels.

increases in cAMP levels are calcium dependent and are blocked by 10^{-5} M *d*-tubocurarine (Table I). Neither dopamine antagonists nor adenosine antagonists alter either basal cAMP levels or the increases in response to nicotine (data not shown), ruling out the possibility that dopamine or adenosine derivatives released from the cells were responsible for the observed increases in cAMP levels via interaction with their respective cell-surface receptors coupled to adenylate cyclase.

Figure 3 illustrates the time course of the effects of NGF on

Figure 2. Dose-response curve for the effects of nicotine on cAMP levels in control and NGF-treated PC12 cell cultures. Cultures were incubated for 2 min in KRH buffer containing the indicated concentrations of nicotine, and cAMP levels were measured as described under "Materials and Methods." Each point represents the mean \pm SE of at least three determinations on separate cultures. NGF-treated cultures were treated for 2 weeks, as in Figure 1. *, significantly different from basal levels ($p < 0.05$); †, significantly different from control cultures ($p < 0.01$).

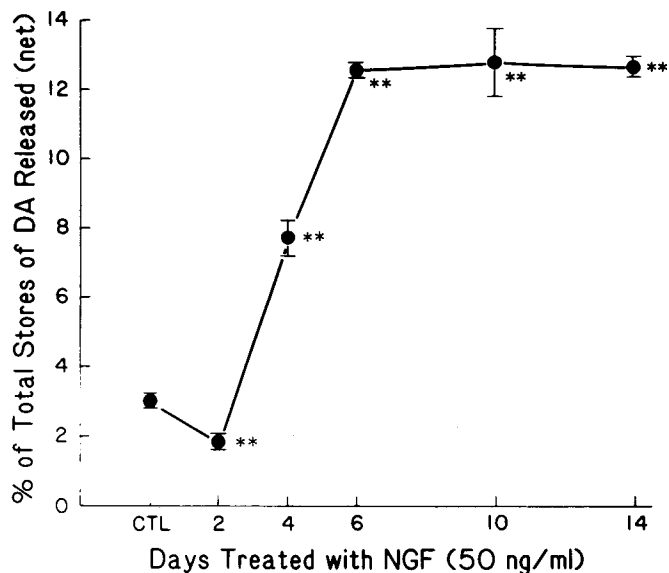
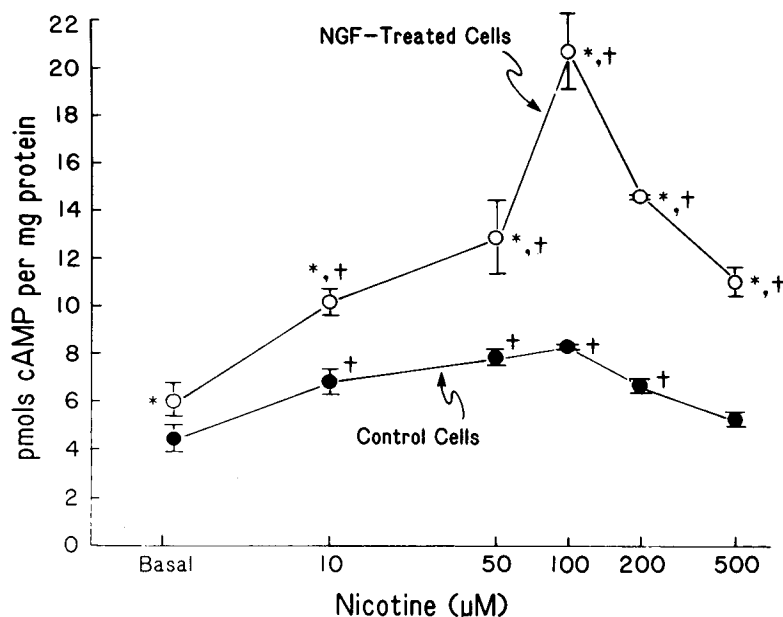


Figure 3. Time course of NGF effects on nicotine-stimulated DA release. Measurements of DA release were performed as described under "Materials and Methods." In all experiments, the nicotine concentration was 10^{-4} M. Cells were inoculated at an initial density of $4 \times 10^4/cm^2$ and cultured for 2 to 14 days in medium supplemented with 50 ng/ml of NGF; medium was changed every 3 days. Each point represents the mean \pm SE of at least three determinations on separate cultures. Release is expressed as net DA release. **, significantly different from control ($p < 0.01$).

nicotine-evoked DA release. Two days after the initiation of treatment, the response is significantly below control levels. The magnitude of the response then increases and reaches its maximal level after 6 days of NGF treatment, prior to extensive neurite outgrowth in the cultures. Two d after initiation of treatment, basal cAMP levels are also significantly lower than control values; they then increase gradually over a period of several d. The increase in cAMP levels in response to nicotine reaches its maximal level after 6 d of treatment (Table II), as does evoked DA release.

Discussion

Nicotine stimulates the release of endogenous DA in PC12 cell cultures, and treatment with NGF enhances this response. These results are similar to those of Greene and Rein (1977b), who studied

TABLE II
Time course of NGF effects on nicotine-stimulated cAMP levels
PC12 cells were treated with NGF as described in Table I, and cAMP levels were measured as described in Figure 2 (legend).

NGF Treatment (days)	Basal	10^{-4} M Nicotine	Relative Increase ^a
Control	4.2 ± 0.6	8.3 ± 0.2^b	1.96
2 (6) ^c	0.8 ± 0.2^d	$1.4 \pm 0.2^{b,d}$	1.75
4 (6)	2.0 ± 0.3^d	$4.9 \pm 0.3^{b,d}$	2.49
6 (7)	3.0 ± 0.4^d	$10.9 \pm 0.7^{b,d}$	3.64
10 (12)	6.1 ± 0.4^d	$21.6 \pm 1.1^{b,d}$	3.56
14 (5)	5.3 ± 0.4^d	$18.7 \pm 0.5^{b,d}$	3.53

^a Level in the presence of nicotine divided by the basal level.

^b Significantly different from basal ($p < 0.01$).

^c Numbers in parentheses, number of determinations on separate cultures.

^d Significantly different from control ($p < 0.05$).

the nicotine-stimulated release of preloaded [³H]norepinephrine in PC12 cell cultures. Additionally, we have determined that nicotine causes increases in cAMP levels that are well correlated in several aspects with DA release. The increases do not appear to be caused indirectly by substances released from the cells. These results suggest that stimulation of the nicotinic cholinergic receptor in PC12 cells results in some manner in the activation of adenylate cyclase. The calcium dependence of the cAMP increase suggests that nicotine stimulates calcium influx into the cells (Ritchie, 1979; Stallcup, 1979). The ensuing rise in intracellular free calcium levels might lead to the activation of a calcium-calmodulin-sensitive adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975) located at some intracellular site. This scheme would explain the inability of Guidotti and Costa (1974) to demonstrate nicotine-mediated stimulation of adenylate cyclase activity in adrenal membrane preparations.

The strong correlation between cAMP levels and DA release lends further support to the notion that cAMP is involved in the process of neurotransmitter release. Additionally, forskolin, a compound which stimulates adenylate cyclase activity in a wide variety of cultured cells (Seamon and Daly, 1983), enhances DA release from PC12 cells (Rabe et al., 1982; Baizer and Weiner, 1985). The exact nature of the involvement of cAMP in the process of release, however, remains to be determined. cAMP may promote the phosphorylation of a contractile or microtubule-associated protein (Sloboda et al., 1975) that might possibly be involved in the translocation of vesicles

to the plasma membrane (Weiner, 1979). Alternatively, cAMP may be involved only indirectly in the release process, either modulating transmembrane calcium fluxes (as in *Aplysia*; Castelucci et al., 1980), or serving to release calcium from intracellular storage sites to promote release. The lack of a strong correlation between cAMP levels and release in some cases (control cells in this study; Baizer and Weiner, 1985; Rabe et al., 1982) suggests that the role of cAMP may in fact be indirect.

The NGF-mediated increases in nicotinic cholinergic responsiveness noted in the present investigation complement the results of other studies which have demonstrated that NGF increases sodium influx (Amy and Bennett, 1983) and membrane depolarizations (Dichter et al., 1977) in response to nicotinic stimulation in PC12 cell cultures. Interestingly, the time course for the effects of NGF on nicotine-stimulated DA release and cAMP levels in the present study is quite similar to that noted by Jumblatt and Tischler (1982) for the NGF-induced increase in muscarinic cholinergic binding sites in these cells. The NGF-induced increase in nicotinic sensitivity noted here may reflect a similar increase in the density of nicotinic receptors in PC12 cells in culture. This increase would presumably be accompanied by an increase in the receptor-associated ion channel, resulting in a greater influx of calcium in response to nicotinic stimulation in the NGF-treated cells, and consequently an enhancement of evoked DA release. Alternatively, NGF treatment may alter calcium metabolism in PC12 cells in such a manner that the release mechanism is more readily activated.

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