0270-6474/81/0110-1085$02.00/0

Printed in U.S.A.

The Journal of Neuroscience
Vol. 1, No. 10, pp. 1085-1095
October 1981

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[1]

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Abstract

The clonal rat pheochromocytoma, PC12, responds to nerve growth factor (NGF) and dibutyryl adenosine cyclic 3′:5′-monophosphate (dbcAMP) by the elevation of cellular protein and RNA levels and the extension of neurites. The simultaneous presence of dbcAMP and NGF produces changes in all three parameters that are additive or greater than the sum of those caused by either agent alone, indicating that the mechanisms by which the two agents act to produce these changes are distinct. The concentration of dbcAMP required for half-maximal stimulation of these changes is also different for each, while NGF is active in all instances at 10^{-11} M. PC12 cells initially generate neurites slowly in response to NGF and, at the same time, develop the capacity to regenerate neurites rapidly, a process termed priming. The cells, however, are not primed by dbcAMP nor does it influence the ability of NGF to prime them. Time lapse cinematography demonstrates that both NGF and dbcAMP each have unique effects on cellular morphology. The latter produces rapid, unstable neurite initiation but does not promote sustained neurite extension. In contrast, NGF has immediate effects at the cell surface with no neurite initiation but later produces sustained neurite outgrowth. Utilizing dbcAMP, it is possible to dissect four morphological responses of PC12 cells to NGF. Three of these may be mechanistically closely tied to occupancy of the NGF plasma membrane receptor.

Nerve growth factor (NGF) is a neuronally acting peptide hormone whose action on the differentiation of neurons within sympathetic and sensory ganglia has been studied extensively (Levi-Montalcini and Angeletti, 1968). While much is known about the effects of NGF on its target cells, the mechanisms which subserve these actions are only now beginning to be understood (Greene and Shooter, 1980). The recent isolation of an NGF-responsive clonal cell line (PC12) from rat pheochromocytoma (Greene and Tischler, 1976) has provided a particularly useful model system in which to explore this problem.

Cultures of PC12 cells respond to the continued presence of NGF by extending neuritic processes and ceasing cell division within 1 week (Greene and Tischler, 1976). This morphological differentiation is a transcriptionally dependent process, presumably requiring the production of gene products which mediate neurite outgrowth (Burstein and Greene, 1978). Once the transcription products are synthesized and present within PC12 cells, neurite outgrowth can proceed in the absence of protein and RNA synthesis and the cells are said to be "primed" (Greene and Tischler, 1976; Burstein and Greene, 1978; Greene, 1978). Recent evidence suggests that the biological role of these gene products involves the reorganization of the cell's cytoskeleton at a regulatory or catalytic level (Gunning et al., 1981a), an idea which is consistent with the failure to detect any NGF-induced preferential changes in the synthesis of the major cytoskeletal proteins in PC12 cells (Garrels and Schubert, 1979).

[1] This work was supported by grants from the National Institutes of Health (NS 04270), the American Cancer Society (BC 325), the National Science Foundation (PCM 792733 to P. C. L.), the Graduate School of the University of Minnesota, and the Minnesota Medical Foundation. P. W. Gunning was supported by a junior fellowship from the California Division of the American Cancer Society and G. E. Landreth was supported by a United States Public Health Service National Research Service award.

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It has been argued that NGF operates by an adenosine cyclic 3′:5′-monophosphate (cAMP)-dependent mechanism in PC12 cells. This derives from two types of experiments. First, it has been reported that NGF induces a transient increase in the intracellular level of cAMP in PC12 cells which, in turn, causes an increase in calcium mobilization (Schubert and Whitlock, 1977; Schubert et al., 1978). However, these two findings have not been reproduced in other laboratories (Hatanaka et al., 1978; Landreth et al., 1980). The second line of evidence relies on the ability of agents which raise intracellular cAMP levels to mimic the biochemical and morphological effects of NGF. In particular, it has been suggested that N^6,O^2-dibutyryl cAMP (dbcAMP) and NGF elicit similar responses from PC12 cells. These responses include (*1*) induction of neurite outgrowth (Schubert and Whitlock, 1977; Schubert et al., 1978), (*2*) increased synthesis of a similar group of proteins (Garrels and Schubert, 1979), and (*3*) increased phosphorylation of the same set of nuclear and cytoplasmic proteins (Halegoua and Patrick, 1980).

Since the weight of evidence for a cAMP-dependent mechanism of NGF action comes from these comparisons, it is critical to demonstrate that the cellular responses are indeed the same and to show that the two agents together produce responses equal to the maximal effect of one agent alone. In only one of the above mentioned responses, phosphorylation of one protein, has it been shown that the maximal combined effect of NGF plus dbcAMP is equal to that of dbcAMP alone (Halegoua and Patrick, 1980). In contrast, it is known that the initiation of neurite outgrowth from PC12 cells in the presence of dbcAMP does not require RNA synthesis, whereas the initiation caused by NGF alone does (Gunning et al., 1981a). Furthermore, it was noted that increases in the cellular RNA concentration and the extent of neurite growth in the presence of both dbcAMP and NGF are equal to at least the sum of the increases due to either agent alone (Gunning et al., 1981a). Hatanaka et al. (1978) also found that addition of NGF to PC12 cells increased the level of ornithine decarboxylase over and above that produced by cAMP alone.

The present studies were undertaken, therefore, to explore these differences in more detail. We report that detailed analysis of NGF-induced changes in cellular RNA and protein levels and cellular morphology are not identical to those induced by dbcAMP. The use of dbcAMP, however, does provide a particularly useful probe with which to dissect a number of discrete steps in the mechanism of NGF action.

Materials and Methods

Cell culture. The PC12 clone of a rat pheochromocytoma was obtained from Dr. David Schubert (The Salk Institute, La Jolla, CA). The cells were grown in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal calf serum and 5% horse serum. Cultures were maintained in a water-saturated atmosphere of 88% air, 12% CO_2. Experiments were performed by replating subconfluent cells at a density of 1 to 2×10^4 cells cm^{-2} and the cells were allowed to reach log phase growth (2 days). At that time, fresh medium was added together with 5 ng/ml of β-NGF (prepared by the method of Smith et al. (1968) and hereafter referred to as NGF) or 1 mM dbcAMP (Sigma) unless otherwise indicated. In some experiments, the fresh medium contained 1 mg/ml of bovine serum albumin (Pentex) either with or without fetal calf serum and horse serum. The effect of dbcAMP has been shown previously to be due to intracellular cAMP and not to the butyrate moiety (Gunning et al., 1981a).

The morphological differentiation of PC12 cells was assayed by determining the percentage of cells with neuritic processes over one cell body diameter in length and possessing a growth cone at their tips. A minimum of 600 cells were examined per dish from randomly chosen fields.

DNA, RNA, and protein determination. The cells were dislodged mechanically from the dishes by trituration and collected by centrifugation at $750 \times g_{av}$ for 5 min. Acid-soluble material was obtained by extracting the cell pellet three times with 0.5 M perchloric acid (PCA) at 4°C. The acid-insoluble material was suspended in 1 M NaOH and incubated at 37°C for 18 hr. A sample of the solution was taken for protein determination by the method of Lowry et al. (1951), using bovine serum albumin (Sigma) as a standard. Concentrated PCA was added to the remainder of the solution to a final concentration of 0.5 M PCA and left at 4°C for 1 hr. The resulting precipitate was collected at $10,000 \times g_{av}$ for 10 min and washed once with 0.5 M PCA. The two supernatants were combined for RNA determination. The pellet was extracted twice with 0.5 M PCA at 80°C for 30 min and the combined supernatants were taken for DNA determination. RNA and DNA were determined by the two-wavelength method of Tsanev and Markov (1960). The amount of DNA was taken as a measurement of cell number with 1×10^6 log phase cells containing 10 μg of DNA. The ratios of RNA:DNA and protein:DNA were taken as a measure of the cellular RNA and protein concentrations. Approximately 95% of PC12 RNA is ribosomal RNA and transfer RNA.

Determination of priming. The extent to which priming occurred and its time course was examined by plating the cells as described previously and adding 50 ng/ml of NGF, 1 mM dbcAMP, or both and changing the medium every other day. Each day, one plate was taken and the percentage of the cells with neurites was determined. The cells then were removed from the plate by trituration and replated into 16-mm wells (30,000 cells/well) in a total volume of 2 ml of culture medium containing 1 ng/ml of NGF. The fraction of cells which regenerated neurites was determined 24 hr later.

Time lapse cinematography. PC12 cells were grown in 25-cm^2 tissue culture flasks (Falcon Plastics) at a density of 1 to 2×10^4 cells/cm^2 in 5 ml total volume for 24 hr. Before filming, the flasks were sealed and removed from the CO_2 incubator to the stage of a Zeiss IM microscope, warmed to 37°C with an Air Stream Incubator (Nicolson Precision Instruments, Inc.). Time lapse cinematography was performed with an Optiquip time lapse apparatus and pictures were taken at frequencies from 15 frames/min to 20 frames/hr. Movies were viewed with a Dynamic Frame analyzer projector (NAC, Inc.).

NGF and dbcAMP were added to flasks during filming sessions by rapidly unsealing the flask and adding 2.5 ml of warmed culture medium containing enough of the agent(s) to raise the concentration of NGF and dbcAMP to 5 ng/ml and 1 mM, respectively. The flasks were resealed immediately. The pH of the culture medium did not change, as indicated by the color of the phenol red in the liquid medium.

Results

The Dose Responses of Dibutyryl Cyclic AMP-induced Effects

Exposure of PC12 cells to NGF results in increases in cellular RNA and protein levels which regulate the extent of neurite growth (Gunning et al., 1981b). Both of these increases have identical NGF dose-response curves with half-maximal increases observed at 0.2 ng/ml and reaching a maximum at 2 to 5 ng/ml. A very similar dose-response relationship is observed for NGF stimulation of neurite regeneration from PC12 cells (Greene, 1977). Although dose-response analysis of *de novo* neurite induction from PC12 cells by NGF cannot be measured accurately because of extensive and rapid degradation of NGF, it is clear that this is also maximal at 2 ng/ml of NGF (see Gunning et al., 1981b). If NGF acts via a cAMP-dependent mechanism, then the dose-response characteristics of dbcAMP-induced changes in these parameters also should be similar.

The following experiments demonstrate that this criterion is not satisfied. Exposure of PC12 cells to dbcAMP for 24 hr resulted in a dose-dependent rise in the cellular RNA concentration (Fig. 1*A*). Half-maximal stimulation was achieved at about 5×10^{-5} M dbcAMP and maximal stimulation was reached between 0.3 and 1.0 mM dbcAMP, resulting in a 30% increase over control sister cultures. NGF, at a concentration which produced maximal stimulation (5.0 ng/ml), resulted in a 15% increase after the same time period (Fig. 1*B*). When NGF (5 ng/ml) was present in the medium, addition of dbcAMP again gave a dose-dependent rise in the cellular RNA concentration (Fig. 1*B*). The increase due to the dbcAMP addition was half-maximal at 4×10^{-5} M and maximal at about 10^{-4} M. The maximal increase due to the addition of dbcAMP was again approximately 30%. Comparison of the data in Figure 1, *A* and *B*, clearly demonstrates that, at all concentrations of dbcAMP studied, the combined effects of a maximal stimulating concentration of NGF (5 ng/ml) plus dbcAMP are at least equal to the sum of their individual effects.

The cellular protein concentration also was measured in these experiments and similar results were obtained. The increase observed in medium containing serum only was half-maximal at about 2×10^{-5} M dbcAMP (Fig. 2*A*), whereas in the presence of 5 ng/ml of NGF, the half-maximal increase due to the dbcAMP addition was obtained already at approximately 10^{-6} M (Fig. 2*B*). In both instances, maximal stimulation was attained at 10^{-4} M. It also should be noted that, whereas no increase in the cellular RNA concentration was detected at 10^{-6} M dbcAMP (Fig. 1*A*), a significant elevation of the cellular protein concentration was observed at this concentration (Fig. 2*A*). This correlates well with the observation that the dbcAMP concentration required, in the presence of NGF, for half-maximal stimulation of the cellular protein was lower than that required for the elevation of cellular RNA. At all dbcAMP concentrations studied, the combined effects of NGF plus dbcAMP were again additive or synergistic (Fig. 2*B*).

The proportion of cells with neurites after 24 hr exposure to dbcAMP was relatively low and the dose-response curve indicated a half-maximal induction at 2×10^{-4} M and a maximal effect at 10^{-3} M (Fig. 3). Exposure of the cells for this period of time to 5 ng/ml of NGF alone gave an even smaller proportion of cells with neurites (about 5%; see Fig. 4*A*). However, the addition of dbcAMP increased the proportion of cells extending neurites in the presence of NGF and the effect was dose dependent (Fig. 3). Half-maximal stimulation occurred at 1.5×10^{-4} M dbcAMP and was maximal at 10^{-3} M (Fig. 3). Concentrations of dbcAMP higher than those shown in Figure 3 produced no further enhancement of neurite outgrowth in either the presence or absence of NGF. The potentiation of NGF-induced neurite outgrowth by dbcAMP clearly gives a much higher proportion of cells with neurites over a wide range of dbcAMP concentrations than do optimal concentrations of either agent alone (see also Fig. 4*A*).

These experiments were made with PC12 cells on plastic, conditions under which relatively few cells grow neurites in the presence of dbcAMP alone. The proportion of cells which respond to dbcAMP with neurite outgrowth was increased to 40% when polylysine was used as the substratum. However, even under these conditions, the marked potentiation of neurite outgrowth by dbcAMP and NGF acting together was still observed.

A summary of the dose-response characteristics of the dbcAMP-induced changes is given in Table I. In the absence of NGF, half-maximal stimulation of protein and RNA levels and of neurite outgrowth, respectively, required increasingly higher concentrations of dbcAMP. An even wider spread of dbcAMP concentrations was observed in the presence of optimal concentrations of NGF.

Priming of PC12 Cells

Exposure of PC12 cells in medium containing 50 ng/ml of NGF resulted in a progressive increase with time in the fraction of cells bearing neurites. Maximum response was approached after 6 to 7 days (Fig. 4*A*). In contrast, 1 mM dbcAMP caused a more rapid appearance of neurites and the response was maximum within 1 day (Fig. 4*A*). NGF ultimately produced a much higher fraction of cells with neurites than did dbcAMP. When the cells were exposed to both agents simultaneously, maximal neurite induction occurred within 1 to 2 days and was not far below that achieved by NGF after 6 days (Fig. 4*A*). In the first 2 days, the response with both agents present was clearly greater than the sum of the responses to either agent alone.

PC12 cells which have been exposed to NGF for 1 week and then had their neurites removed by trituration regenerate neurites within 24 hr of re-exposure to NGF (Greene, 1977). As shown in Figure 4*B*, the acquisition of

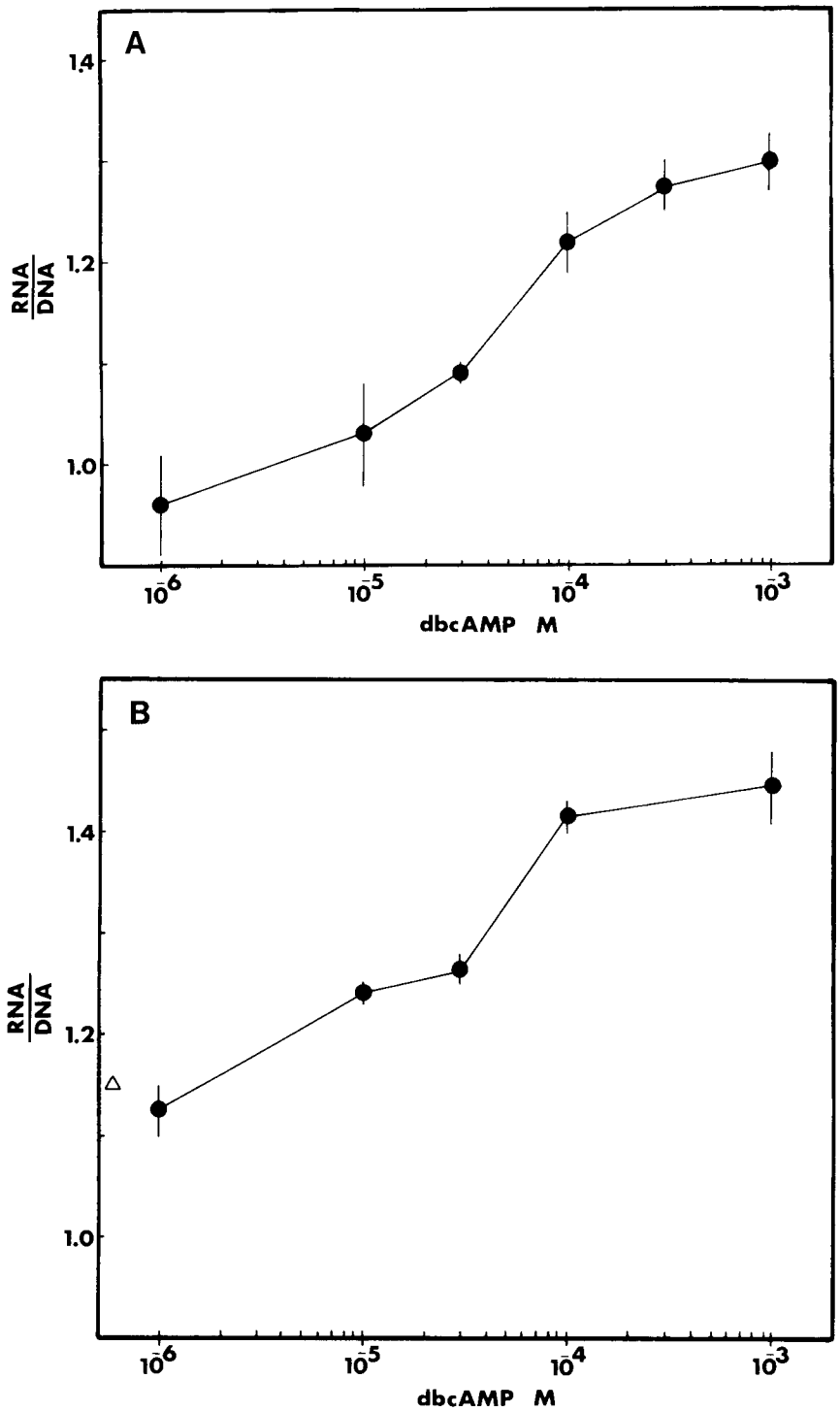


Figure 1. Dose-responsive increases in PC12 cellular RNA levels as a function of dbcAMP concentration. PC12 cells were plated at 1 to 2 × 10^4 cells cm^{-2} and, after 2 days, the indicated concentrations of dbcAMP were added together with fresh medium plus serum (*A*) or with medium plus serum plus 5 ng/ml of NGF (*B*). After 24 hr, the cellular RNA concentration was measured in terms of the RNA/DNA mass ratio. The resulting values are expressed relative to control sister plates which received only fresh medium plus serum. The stimulation over controls due to the presence of 5 ng/ml of NGF alone is shown (△) in *B*. The values are the means of three individual plates and the *bars* represent the SD.

the ability to regenerate neurites rapidly, termed "priming" (Burstein and Greene, 1978), was dependent upon the time of exposure to NGF (see also Greene, 1977). The time course of priming closely paralleled the time course of the initial generation of neurites by NGF (cf., Fig. 4, *A* and *B*). In contrast, dbcAMP alone did not prime the cells (Fig. 4*B*), although short neurites were observed when the cells were cultured in the presence of dbcAMP

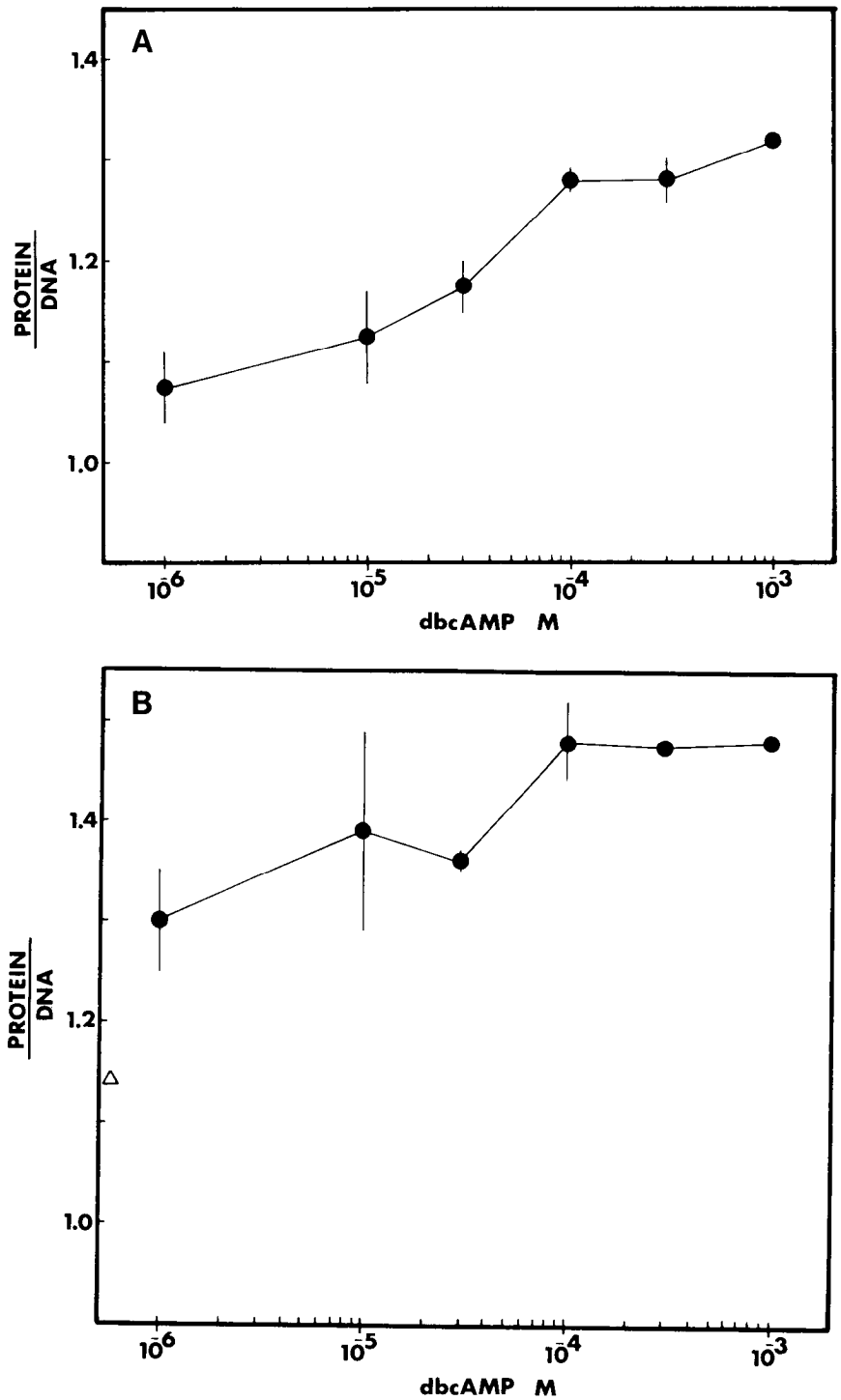


Figure 2. Dose-responsive increases in PC12 cellular protein levels as a function of dbcAMP concentration. The experiment was performed and the results are expressed as described for Figure 1. The cellular protein concentration was measured in terms of the protein/DNA mass ratio. The dbcAMP was added with fresh medium plus serum (*A*) or with medium plus serum plus 5 ng/ml of NGF (*B*). The stimulation over controls due to the presence of the 5 ng/ml of NGF alone is shown (△) in *B*.

(Fig. 4*A*). Furthermore, the simultaneous presence of NGF plus dbcAMP did not result in a faster rate of priming than that achieved by NGF alone (Fig. 4*B*). This is in marked contrast to the observation that a large fraction of cells possessed neurites after a short exposure to NGF plus dbcAMP (Fig. 4*A*). It is therefore apparent that, while dbcAMP markedly potentiates the initial appearance of neurites, it neither endows the cells with a capacity to regenerate their neurites nor influences the rate at which NGF induces the capacity. Furthermore,

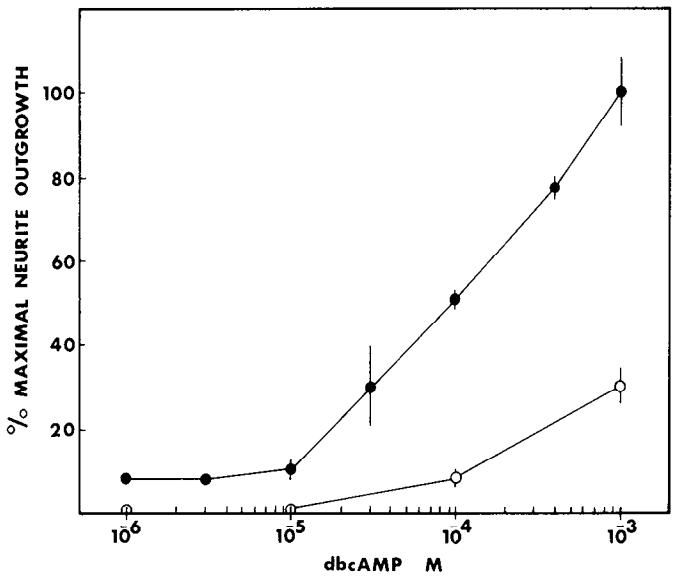


Figure 3. Dose-responsive increases in neurite outgrowth from PC12 cells as a function of dbcAMP concentration. PC12 cells were plated at 1 to 2×10^4 cells cm^{-2} and, after 2 days, the indicated concentrations of dbcAMP were added together with fresh medium plus serum (○——○) or with medium plus serum plus 5 ng/ml of NGF (●——●). After 24 hr, the extent of neurite outgrowth was measured and is expressed as the mean percentage of the maximal outgrowth from three individual plates, with the *bars* showing the SD. The value for NGF alone was 8% of maximal outgrowth. No neurites were observed in control sister cultures.

TABLE I

Dose-response characteristics of dbcAMP-elicited increases in protein and RNA levels and neurite outgrowth in the presence of NGF

Agent	Concentration of dbcAMP Required to Elicit Half-maximal Response in		
	Protein / DNA	RNA / DNA	Neurite Outgrowth
	M		
dbcAMP	2×10^{-5}	5×10^{-5}	2×10^{-4}
dbcAMP + NGF[a]	10^{-6}	1×10^{-5}	1.5×10^{-4}

[a] Added at 5 ng/ml.

whereas the "priming" of cells is coupled to the initiation of neurite outgrowth in the presence of NGF, these two events become uncoupled in the presence of dbcAMP.

Stability of Neurites Induced in the Presence of dbcAMP

Removal of dbcAMP from the medium surrounding cells exposed to this agent for 24 hr resulted in a rapid loss of neurites (Fig. 5*A*). The half-life of this loss was about 30 min. A similar rapid loss was seen with cells which were cultured in the presence of both NGF and dbcAMP for 24 hr. Removal of dbcAMP from the medium resulted in a loss of neurites with a half-life of 40 min (Fig. 5*B*). When the NGF was removed instead of dbcAMP, the half-life for loss of neurites was between 1 and 2 hr and the percentage of cells with neurites dropped to a level close to that maintained by dbcAMP alone (Fig. 5*C*). The removal of both agents produced a neurite loss with a half-life of about 30 min (Fig. 5*D*).

Two important points emerge from this experiment. First, neurites induced in the presence of dbcAMP for 24 hr are inherently unstable and are absolutely dependent upon the continued presence of this agent for their survival. The half-life of the mechanism by which dbcAMP achieves this appearance of neurites is less than 30 to 40 min. Secondly, the potentiation of the appearance of dbcAMP-induced neurites by NGF is also a transitory phenomenon with a half-life of 1 to 2 hr.

Morphological Responses to NGF and dbcAMP

Differences in the morphological responses of PC12 cells to NGF and to dbcAMP were examined with time lapse microcinematography. Each agent stimulated distinct short term morphological responses in addition to the long term effects on neurite production reported above. Combination of the two agents produced short term morphological responses typical of each agent and induced a degree of neurite formation which exceeded the effects of either drug alone.

PC12 activity in the absence of NGF and dbcAMP

In medium without these agents, it was observed that PC12 cells were grossly rounded and did not migrate far upon the *in vitro* substratum. At higher magnification, filopodia and small lamellipodia protruded continuously over the substratum beyond the rounded cell margin. Usually several limited portions of the cell periphery exhibited protrusive activity, while the remainder of the cell periphery appeared smoothly curved with no obvious protrusions beyond the rounded cell margin. Occasionally, cells had many small blebs, less than 1 μm across, which protruded and regressed rapidly from many points of the cell.

Response to NGF

Addition of 5 ng/ml of NGF to the culture medium stimulated an immediate increase in the protrusive activity of PC12 cells (Fig. 6, *a* to *c*). Within 10 min, the motile portions of the cell periphery were more prominent and numerous, showing primary responses of an increased number and length of filopodia and lamellipodia and a broadening of the motile portions of the cell periphery. These responses recall the rapid changes in surface morphology seen in the scanning electron microscope (Connolly et al., 1979). Although the intense initial protrusion subsided after approximately 20 to 25 min (Fig. 6, *d* and *e*), an increased protrusion from the cell margin persisted for 1 hr or more after adding NGF.

In spite of this stimulated motility, PC12 cells did not extend neurites until many hours after the addition of NGF. Cells retained several areas of surface protrusion, but the formation of neurites became common only after 70 hr or more of NGF treatment. The number of cells with neurites increased over the next 30 to 50 hr. In most cases, neurites were initiated from peripheral sites which had been active in protrusion for several hours.

Response to dbcAMP

The immediate response to dbcAMP was quite different from the response to NGF. Within 10 min of adding 1 mM dbcAMP, neurites, characterized by terminal growth cones and distinct cylindrical shafts, sprouted

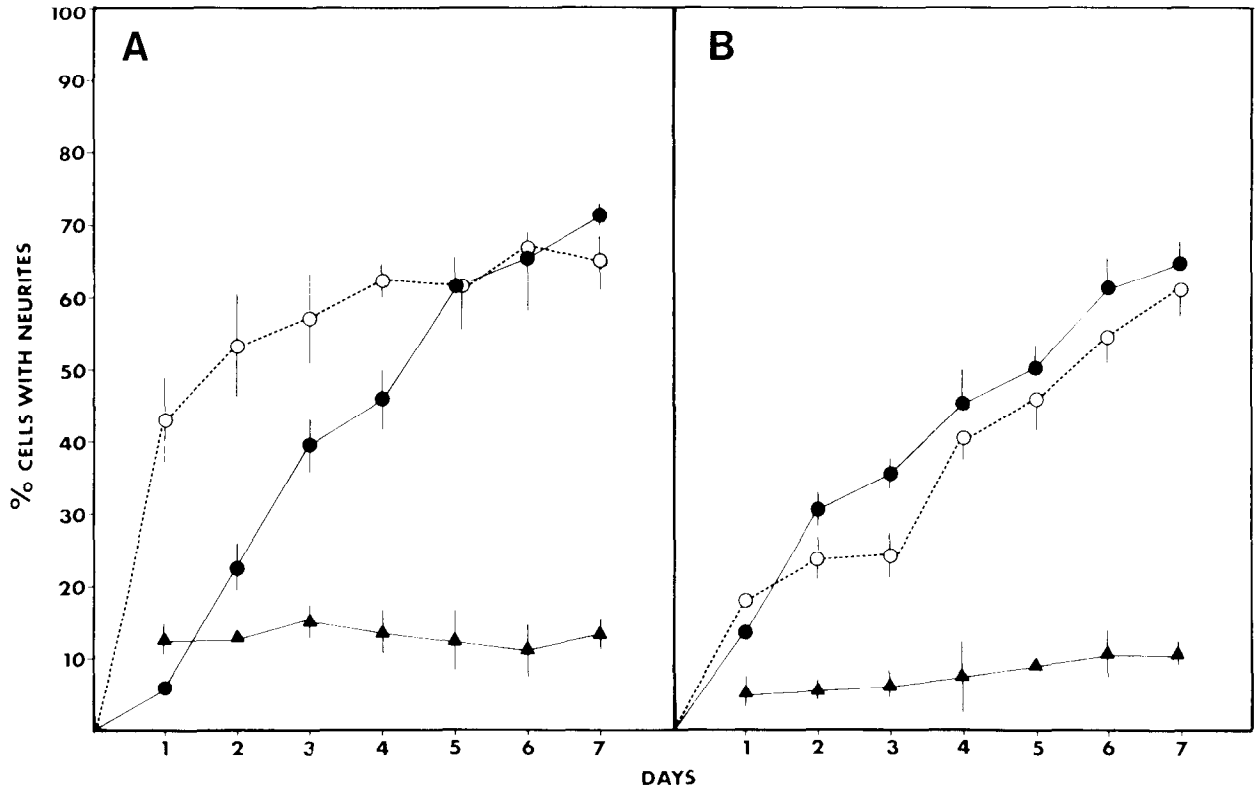


Figure 4. The effect of NGF and dbcAMP on neurite outgrowth and priming. *A*, PC12 cells were cultured in the presence of NGF (50 ng/ml, ●——●), dbcAMP (1 mM, ▲——▲), or both agents (○- - -○) and the fraction of neurite-bearing cells was determined on each day of culture. *B*, The PC12 cells were cultured with the appropriate agent as indicated in *A* for the period shown. The cells were washed, divested of their neurites by trituration in medium plus serum, and replated in 16-mm wells as described under "Materials and Methods." NGF was added (1 ng/ml) and the fraction of neurite-bearing cells was determined 24 hr later. The data represent the mean ± SD of triplicate determinations performed on each of duplicate samples.

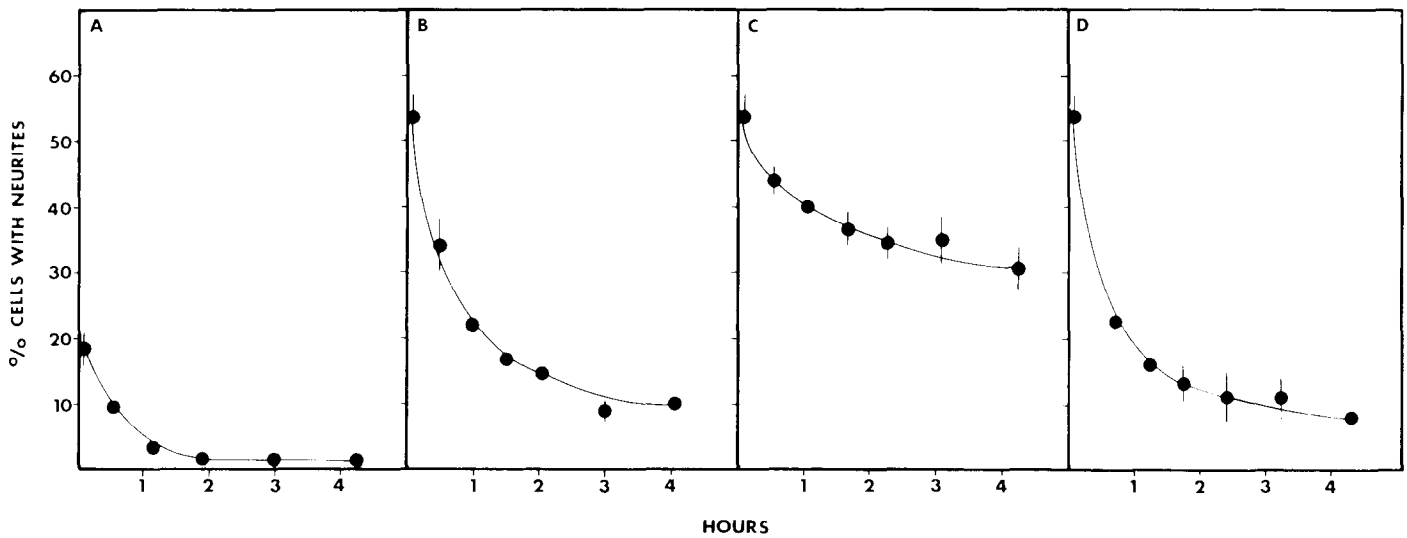
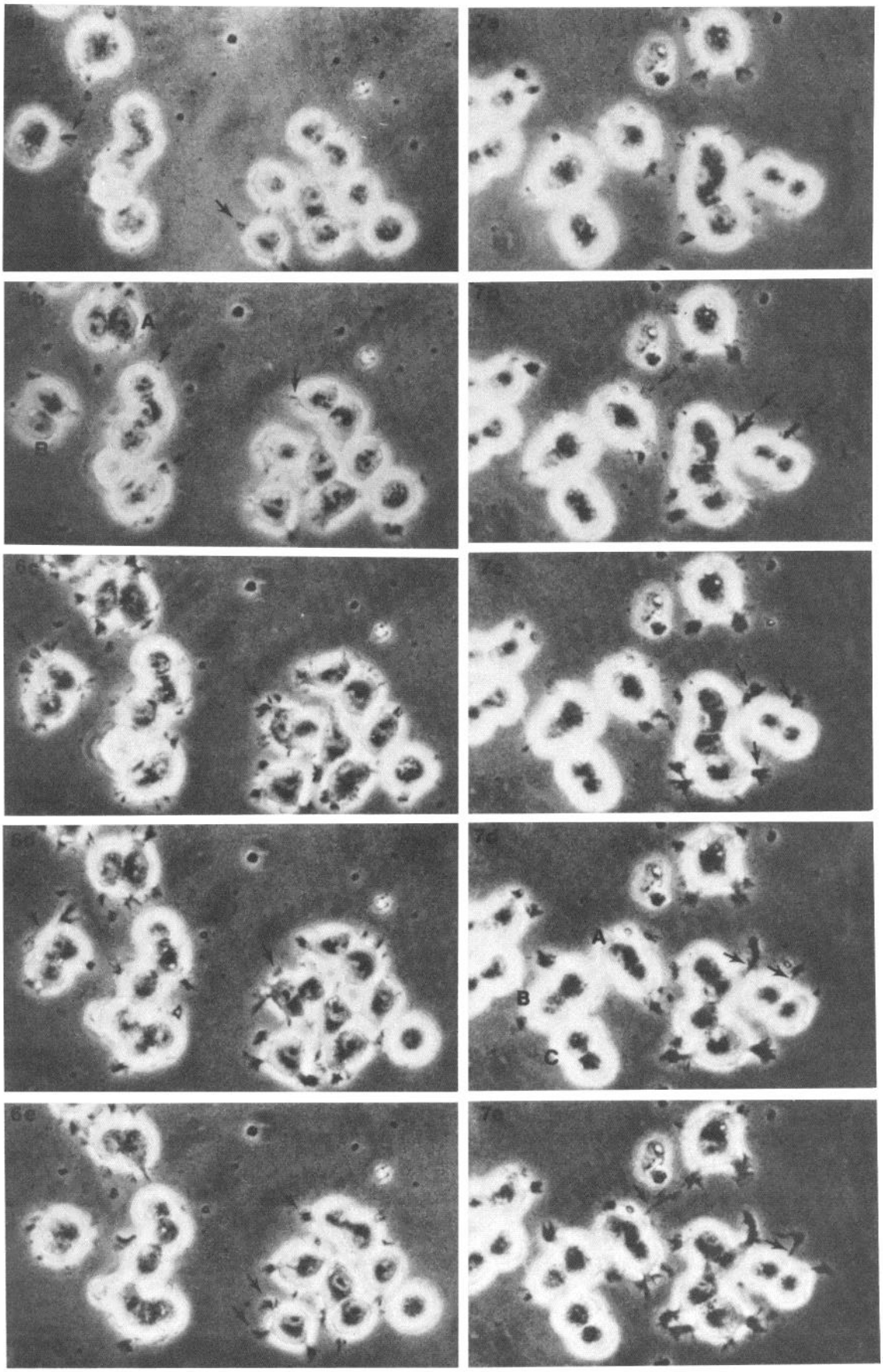


Figure 5. Stability of neurites produced by NGF and dbcAMP. PC12 cells were treated with dbcAMP (1 mM) (*A*) or dbcAMP (1 mM) and NGF (50 ng/ml) (*B*, *C*, and *D*) for 24 hr. The loss of neurites was followed by the determination of the fraction of neurite-bearing cells at various times after the removal of dbcAMP (*A* and *B*), NGF (*C*), or both agents (*D*) from the culture medium. The data represent the mean ± SD measured in triplicate cultures with 200 cells counted per culture.

from many cells (Fig. 7, *a* to *c*). These neurites continued to grow (Fig. 7, *d* and *e*), reaching 15 to 30 μm in the first 4 to 6 hr after adding dbcAMP. However, they did not lengthen further over the subsequent 48 hr or longer. Many neurites regressed totally, and the cells resumed a rounded shape with several peripheral sites of protrusion. Thus, after 24 hr in dbcAMP and for 90 hr of observation, few PC12 cells had actively growing neurites.

At higher magnification, it was apparent that the rapid neurite sprouting by dbcAMP-treated PC12 cells was not


6b
7b
6c
A
B
7c
6d
7d
6e
7e
A
B
C

accompanied by enhanced protrusive activity at the cell margin, as when NGF was added. Rather, neurites originated from protrusive regions of the cell margin, as though these regions had transformed into growth cones. This sequence resembles neurite initiation by embryonic ciliary neurons *in vitro*. In fresh medium, ciliary neurons protrude filopodia from many points of their perimeters, but, when heart-conditioned medium is used, neurites sprout from one or more of these protrusive sites, while the remainder of the cell margin becomes smooth and quiescent (Collins, 1978).

Treatment with both dbcAMP and NGF

In order to assess the response evoked by one agent in the presence of the other, PC12 cells were treated first with one agent and, once its effects had occurred, the other agent was added. Other than the observations noted below, the order of the addition did not alter the net results that the combination of NGF and dbcAMP induced a more rapid neurite differentiation than did either agent alone (Fig. 5*A*).

First dbcAMP, then NGF plus dbcAMP. PC12 cells were exposed to 1 mM dbcAMP for 24 hr when about 10% of the cells had short neurites. At this point, 5 ng/ml of NGF was added, and the expected stimulation of protrusion followed, both at the neurite growth cones and at the cell somas. There was no immediate spurt of neurite formation. Instead, during the next 2 to 24 hr, many cells initiated neurites, and all neurites grew slowly but steadily until filming ended at 100 hr. At approximately 70 to 80 hr after NGF was added, the rate of elongation of many of the neurites began to increase.

First NGF, then dbcAMP plus NGF. PC12 cells were treated with 5 ng/ml of NGF for 24 hr, a time when no cells had neurites. At this point, 1 mM dbcAMP was added. Many cells formed neurites within a short time, as when dbcAMP was added to previously untreated cells. Neurite elongation continued slowly over the subsequent 48 hr. None of the regression of neurites exhibited by cells treated with dbcAMP alone was seen. As noted in the preceding paragraph, an increase in the rate of neurite elongation became apparent at a time corresponding to 70 to 80 hr after the addition of NGF.

NGF and dbcAMP simultaneously. When both agents were added together, responses typical of each were noted. Protrusive activity was stimulated immediately and neurites sprouted from many cells, often from preexisting protrusive sites. However, after the initial burst of neurite initiation, the rate of elongation lagged and remained slow for many hours.

Discussion

Comparison of the action of dbcAMP and NGF. The data presented in this paper demonstrate that dbcAMP does not mimic all of the actions of NGF on PC12 cells and, furthermore, that the mechanisms of dbcAMP and NGF action are distinguishable for all of the parameters used in this study. This conclusion is based on the following evidence: (*1*) dbcAMP-induced changes in cellular RNA and protein levels and dbcAMP induction of neurites occur at widely different concentrations of dbcAMP, suggesting that several separate pathways are being stimulated sequentially by successively higher concentrations of this agent. In contrast, increases in these same parameters elicited by NGF alone show almost identical dose-response characteristics (Gunning et al., 1981a, b; Greene, 1977), consistent with the stimulation of a single pathway (Figs. 1 to 3); (*2*) at all concentrations of dbcAMP, the increases in these three parameters induced by dbcAMP together with a maximally stimulating concentration of NGF are equal to or greater than the sum of the effects of both agents acting alone (Figs. 1 to 3); (*3*) Unlike NGF, dbcAMP cannot prime PC12 cells (Fig. 4*B*); (*4*) whereas, the induction of neurites by NGF is tightly coupled to priming, the presence of dbcAMP uncouples these two events; furthermore, dbcAMP does not influence the rate at which NGF primes the cells (Fig. 4); (*5*) the rapid changes in cell surface morphology induced by dbcAMP are entirely distinct from those elicited by NGF (Figs. 6 and 7); (*6*) when both NGF and dbcAMP are added simultaneously, the rapid changes in morphology are typical of both agents alone; (*7*) although dbcAMP induces the initiation of neurite outgrowth, it does not result in continued neurite growth and many neurites totally regress. In contrast, initiation of neurite outgrowth induced by NGF is tightly coupled to continuous neurite growth.

In addition, there is the recent demonstration that,

Figure 6 (*left*). Frames from a time lapse film showing the response of PC12 cells to the addition of 10 ng/ml of NGF to the culture medium. All magnifications × 800. *a*, Just before adding NGF. Many cells have limited areas of protrusive activity at their periphery (*arrows* indicate two). *b*, 2 min after adding NGF. Response to the hormone is already visible and seen as new protrusive sites at the cell margins (*arrows*). Additional cells seen at *points A* and *B* are the result of a rapid movement of cells on the substratum so that individuals are seen more easily. *c*, 8 min after adding NGF. Stimulation of protrusion is maximal, and broad areas of motile activity at the cell margin are seen (*arrows*). *d*, 25 min after adding NGF. Protrusive activity induced by NGF has begun to subside. Compare sites marked by *arrows* to activity in *c*. *e*, 45 min after adding NGF. Protrusion continues to decrease but is still enhanced compared to activity before NGF was added. Compare activity at *arrows* to same sites in *a*.

Figure 7 (*right*). Frames from a time lapse film showing the response of PC12 cells to the addition of 1 mM dbcAMP to the medium. All magnifications × 800. *a*, Just before adding dbcAMP. Many cells have limited areas of protrusive activity. *b*, 5 min after adding dbcAMP. Response to dbcAMP is seen as protrusion of the cell periphery (*arrows*). *c*, 10 min after adding dbcAMP. Several prominent protrusions are seen (*arrows*), but motile activity is not stimulated to the extent that NGF induces (compare to Fig. 6*c*). *d*, 22 min after adding dbcAMP. Neurites have been initiated at several points. Thin shafts of initiated neurites are not formed at these times by NGF-treated cells. Movement of cells on the substratum at *points A*, *B*, and *C* makes the presence of two cells evident. *e*, 40 min after adding dbcAMP. Neurites have elongated further (*arrows*). The sequence of stimulated and then reduced protrusion does not occur following the addition of dbcAMP.

unlike NGF, dbcAMP induction of neurites is not RNA synthesis dependent and that the neurites induced by dbcAMP cannot be maintained by NGF (Gunning et al., 1981a). While these results suggest that NGF does not act through a cAMP-dependent mechanism, they do not exclude a role for cAMP in the mediation of NGF action.

Dissection of NGF action on PC12 cells. The action of dbcAMP on PC12 cells endows the cells with a number of properties characteristic of primed cells, albeit in an unstable state. In particular, stimulation of neurite outgrowth by NGF in the presence of dbcAMP is RNA synthesis independent (Gunning et al., 1981a). This is characteristic of NGF action on primed cells in contrast to that of naive cells (Burstein and Greene, 1978). NGF also stimulates neurite outgrowth from PC12 cells treated with dbcAMP at a much faster rate than it does from untreated naive cells. Again, this is a characteristic of NGF action on primed cells. Thus, dbcAMP allows an examination of the effect of NGF on neurite outgrowth in conditions where the latter is uncoupled from the NGF-induced priming event.

A close relationship between the occupancy of the NGF plasma membrane receptor and some morphological responses is suggested by these experiments. When NGF is removed from cells exposed to NGF plus dbcAMP for 24 hr, there is a loss of NGF-stimulated neurite appearance with a half-life of 1 to 2 hr. It is known that NGF only slowly dissociates from these cells when it is removed from the medium, with a half-life also measured in hours (Landreth and Shooter, 1980). These results suggest that the NGF stimulation of neurite initiation and growth in the presence of dbcAMP is mechanistically closely linked to occupancy of the NGF plasma membrane receptor. It should be noted that a close temporal relationship between neurite morphology and NGF plasma membrane receptor occupancy has been demonstrated recently for sensory neurons grown in culture (Griffin and Letourneau, 1980). A similar conclusion may be drawn from the rapid changes that occur in PC12 cell morphology following the initial exposure to NGF (Connolly et al., 1979; this paper).

The existence of four distinct morphological responses of PC12 cells to NGF also can be discerned from these studies. The first occurs immediately upon addition of NGF and results in rapid cell surface protrusive activity. The second involves the initiation of neurite outgrowth and is normally tightly coupled to the RNA synthesis-dependent priming of the cells. The relationship between priming and initiation is uncoupled by dbcAMP. This suggests that two complementary mechanisms are being triggered by NGF to initiate neurite outgrowth and that dbcAMP can circumvent the requirement for the RNA synthesis-dependent step. The third, early neurite growth which occurs after neurite initiation, is normally tightly coupled to the latter, but the use of dbcAMP allows this response to be observed uncoupled from neurite initiation. Finally, NGF causes an elevated rate of neurite growth after 70 to 80 hr exposure of the cells. The first three of these phenomena appear to be mechanistically closely tied to the occupancy of the NGF plasma membrane receptor. However, this is not true for the priming response nor for the increased rate of neurite growth at later times.

The agent dbcAMP is thus a useful probe with which to explore the mechanism of action of NGF. The rapid reversibility of the morphological response of PC12 cells to dbcAMP is similar to the dbcAMP induction of cell shape changes in Chinese hamster ovary cells (Hsie and Puck, 1971; Porter et al., 1974; Puck, 1977). The recent study of Lawrence et al. (1979) is also of particular interest in this regard since it showed that exposure of rat ovarian granulosa cells to follicle-stimulating hormone caused a transient, dose-dependent increase in intracellular cAMP levels accompanied by an alteration in cell morphology. The reversal of the morphological response of the granulosa cells lagged about 1 to 2 hr behind the fall in intracellular cAMP, suggesting that the cAMP-dependent mechanism responsible for this event has a half-life of 30 to 60 min.

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