

The Role of cAMP in Nerve Growth Factor-promoted Neurite Outgrowth in PC12 Cells

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Abstract. Nerve growth factor (NGF)-mediated neurite outgrowth in rat pheochromocytoma PC12 cells has been described to be synergistically potentiated by the simultaneous addition of dibutyryl cAMP. To elucidate further the role of cAMP in NGF-induced neurite outgrowth we have used the adenylate cyclase activator forskolin, cAMP, and a set of chemically modified cAMP analogues, including the adenosine cyclic 3',5'-phosphorothioates (cAMPS) (Rp)-cAMPS and (Sp)-cAMPS. These diastereomers have differential effects on the activation of cAMP-dependent protein kinases, i.e., (Sp)-cAMPS behaves as a cAMP agonist and (Rp)-cAMPS behaves as a cAMP antagonist. Our data show that the establishment of a neuritic network, as observed from PC12 cells treated with NGF alone, could not be induced by either forskolin, cAMP, or cAMP analogues alone. The presence of

NGF in combination with forskolin or cAMP or its agonistic analogues potentiated the initiation of neurite outgrowth from PC12 cells. The (Sp)-cAMPS-induced stimulation of NGF-mediated process formation was successfully blocked by the (Rp)-cAMPS diastereomer. On the other hand, NGF-stimulated neurite outgrowth was not inhibited by the presence of the cAMP antagonist (Rp)-cAMPS. We conclude that the morphological differentiation of PC12 cells stimulated by NGF does not require cAMP as a second messenger. The constant increase of intracellular cAMP, caused by either forskolin or cAMP and the analogues, in combination with NGF, not only rapidly stimulated early neurite outgrowth but also exerted a maintaining effect on the neuronal network established by NGF.

THE polypeptide hormone nerve growth factor (NGF)¹ is involved in the development and maintenance of sympathetic and certain sensory neurons (21) and is also required for the growth, survival, and differentiation of primary cultures of sympathetic neurons (35). Although many effects of NGF in its target cells have been extensively studied, its molecular mechanisms of action are far from understood (8). The PC12 clonal line of rat pheochromocytoma cells has become a useful model system for studying the mechanism of action of NGF (9). PC12 cells respond to NGF by acquiring many properties of sympathetic neurons; among these, they extend long neuritic processes and cease cell division (9). The morphological differentiation to the neuron-like phenotype, e.g., the initiation of neurite outgrowth, is a transcription-dependent process and presumably requires the synthesis of gene products (3). PC12 cells that have been exposed to NGF for the first time require up to a week until most of the cells have extended neuritic processes (9). However, a "priming mechanism" has been described. When PC12 cells pretreated with NGF for 7–10 d are divested of their processes by mechanical disruption and then replated in the presence of

NGF they will regenerate their neurites within 24 h (3, 10). This neurite regeneration, as opposed to neurite initiation from "naive" cells, does not require RNA or protein synthesis (3). Thus, Greene et al. (10) have suggested that priming of PC12 cells causes the specific stimulation of a transcription-dependent synthesis and accumulation of material which enables the cells to produce neuritic processes.

It has been discussed that the NGF-responses in PC12 cells are mediated through cAMP. This assumption was mainly based on the observations that (a) NGF increased cAMP content in PC12 cells during the first minutes after exposure (32, 33); (b) cAMP and NGF modulate the synthesis of the same set of proteins (6); and (c) dibutyryl cAMP (Bt₂cAMP) and NGF produce basically the same phosphorylation patterns in PC12 cells (15, 29, 38). However, PC12 cells cannot be primed by Bt₂cAMP (10, 14, 16), and NGF-induced changes in cellular RNA and protein levels and cellular morphology were different from those induced by Bt₂cAMP (13, 14). In addition, the induction of a high molecular weight microtubule-associated phosphoprotein was regulated only by NGF and not by Bt₂cAMP (12). Thus, Greene et al. (11) were the first to suggest that NGF and cAMP elicit neurite outgrowth by different mechanisms.

To clarify further the role of cAMP in NGF-induced neurite outgrowth from PC12 cells we have used a set of selected

¹ Abbreviations used in this paper: 8-BrcAMP, 8-bromoadenosine cAMP; Bt₂cAMP, dibutyryl cAMP; 2'-O-Bt-cAMP, 2'-O-monobutyryl cAMP; 6-ClcAMP, 6-chloropurineriboside; GAB, 1-N-oxide-adenosine cAMP; HPLC, high-performance liquid chromatography; NGF, nerve growth factor; 8-PCTP, 8-p-chlorophenylthio cAMP.

chemically modified cyclic nucleotides (17, 19). We have also applied the (Rp) and (Sp) diastereomers of adenosine 3',5'-phosphorothioate (cAMPS) (Fig. 1), which are known to act differentially on the activation of cAMP-dependent protein kinases (4, 5, 24). (Sp)-cAMPS had an activating effect on cAMP-dependent protein kinases (types I and II), whereas the highly purified (Rp)-cAMPS isomer did not activate but rather inhibited protein kinases, thus behaving as a cAMP antagonist (28, 30, 31). Therefore, (Rp)-cAMPS provides a useful means with which to investigate cAMP-mediated effects in neuronal differentiation of cultured cells. In addition, we have studied the effect of forskolin, a diterpene known as a potent activator of adenylate cyclase (34), on the morphological differentiation of PC12 cells.

Materials and Methods

Forskolin was purchased from Calbiochem-Behring (Frankfurt, FRG). cAMP and its analogues were from Boehringer (Mannheim, FRG). (Rp)-cAMPS and (Sp)-cAMPS were synthesized in our laboratory as described before (1, 18, 22, 23). All analogues used contained <0.5% cAMP as determined by high-performance liquid chromatography (HPLC). The (Rp)-cAMPS derivative was specifically purified and contained <0.01% of either cAMP or the (Sp)-cAMPS diastereomer. Tissue culture media were from Boehringer.

Reversed-Phase HPLC

HPLC for determining cyclic nucleotides in the growth medium was carried out as described by Braumann and Jastorff (2). PC12 cells were cultured on 35-mm-diam plates (2×10^5 cells/dish) and (Sp)- or (Rp)-cAMPS (1 mM) was added at day 0. Aliquots of the cell supernatant were analyzed every 24 h.

Cell Culture

PC12 cells (passages 30–40) were grown as described by Greene and Tischler (9) on collagen-coated tissue culture dishes either with or without NGF (50 ng/ml) in complete medium consisting of 85% RPMI 1640, 10% horse serum, and 5% fetal calf serum. The content of confluent culture (10-cm diam) was transferred to 20 plates (35-mm diam), and fresh medium containing cAMP analogues or forskolin as indicated was added 24–48 h after plating. Growth medium supplemented with the compounds was added every 2–3 d. Cells were viewed and photographed using a Zeiss ICM 405 inverted microscope with an automatic camera.

Quantitation of Neurite Outgrowth

The morphological differentiation of PC12 cells was assayed by determining the percentage of neurite-bearing cells. Subconfluent cultures were recultured at $1-2 \times 10^4$ cells per cm^2 . After 1–2 d fresh medium supplemented with NGF or/and cAMP or its analogues was added. Culture medium was changed every 2–3 d with the addition of fresh NGF and cAMP derivatives. Small clumps with neuritic processes longer than one cell body diameter were scored. A minimum of 300 clumps per culture were examined by strip counting. All experiments were performed at least three times. Variations were <5%.

Results

Effects of cAMP Analogues on Neurite Formation

In PC12 cells the initiation of neurite outgrowth could be potentiated by the simultaneous addition of Bt_2cAMP and NGF (11, 13, 39). Most of the studies on the role of cAMP in NGF-induced neurite outgrowth have been carried out by using Bt_2cAMP , a double-modified cAMP analogue. Bt_2cAMP has to be metabolically activated before binding to cAMP receptor proteins (20), and butyric acid is released during the metabolic activation. To exclude cAMP nonrelated unspecific effects caused by the metabolites, we have used a set of chemically modified cAMP derivatives that directly bind to protein kinases, mainly: 2'-O-monobutyryl cAMP (2'-

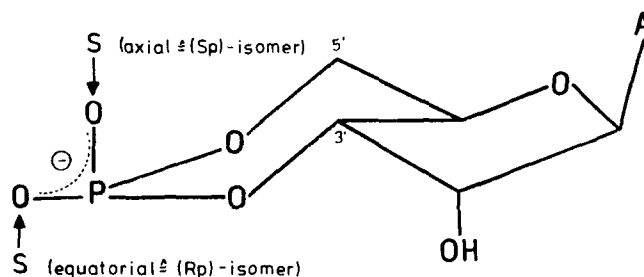


Figure 1. Chemical structure of the phosphorothioate analogues of cAMP.

0-BtcAMP), 6-chloropurineriboside cAMP (6-ClcAMP), 8-bromoadenosine cAMP (8-BrcAMP), 8-*p*-chlorophenylthio cAMP (8-PCTP), 1-N-oxide-adenosine cAMP (GAB), adenosine-3',5'-phosphorothioates (Sp) and (Rp) [(Sp)-cAMPS and (Rp)-cAMPS] (Fig. 1), and, for comparison, Bt_2cAMP . The exposure of PC12 cells to cAMP analogues at concentrations of up to 10^{-3} M did not produce neurite formation, yet some of the derivatives, e.g., Bt_2cAMP , 6-Cl cAMP (Fig. 2), GAB, and (Sp)-cAMPS (not shown), initiated the production of short processes, as has been described for Bt_2cAMP before (13, 16). However, these processes did not continue to grow to form a network, even when cells were treated with high concentrations of cAMP analogues (10^{-3} M) for up to 6 to 8 d (Fig. 3, *b* and *c*). NGF (50 ng/ml) initiated neurite outgrowth in PC12 cells progressively. After 2 d only 20–30% of the cells were neurite bearing, and maximal response was achieved only after 6–8 d in culture (Fig. 2) (9). The simultaneous addition of NGF and cAMP analogues caused a more rapid increase in neurite initiation than did NGF alone (Fig. 2, *d-i*).

All analogues known to act as cAMP agonists in combination with NGF induced a degree of early neurite formation by far exceeding the effect of NGF or cAMP analogues alone. As shown in Fig. 4 after 2–3 d 50–80% of the cells had produced long neurites, and elaborated a branched neuronal network (Fig. 2, *e-i*). Treatment with GAB was revealed to be toxic after 2–3 d, and the application of 8-PCTP to low density cultures caused the cells to clump, making impossible the quantitative evaluation beyond day 3 in culture (Fig. 4, *C*). In cultures treated with NGF and 8-BrcAMP many giant cells with long branched processes were observed (Fig. 2*h*, center). The addition of cCMP or cGMP analogues to the NGF-containing culture medium did not cause marked stimulation of process outgrowth, as shown for 8-BrcGMP in Fig. 4*B*. Half maximal stimulation of neurite outgrowth occurred at $\sim 2 \times 10^{-6}$ M for 2'-O-monobutyryl cAMP, and 2×10^{-5} M for dibutyryl cAMP and (Sp)-cAMPS, maximal effects were reached at 10^{-3} M for all three agents.

Effect of cAMP

cAMP analogues, especially Bt_2cAMP , are widely used in cell biology since it is believed that Bt_2cAMP more easily penetrates cell membranes and is more resistant to hydrolysis by cAMP phosphodiesterase than cAMP itself (25). We have added cAMP to the growth medium of PC12 cells and found that cAMP in combination with NGF had the same synergistic effect on early neurite outgrowth as shown above for its analogues (Fig. 4*A*). After 2 d in culture in the presence of cAMP (10^{-3} M) and NGF, 80% of the cells exerted neuritic

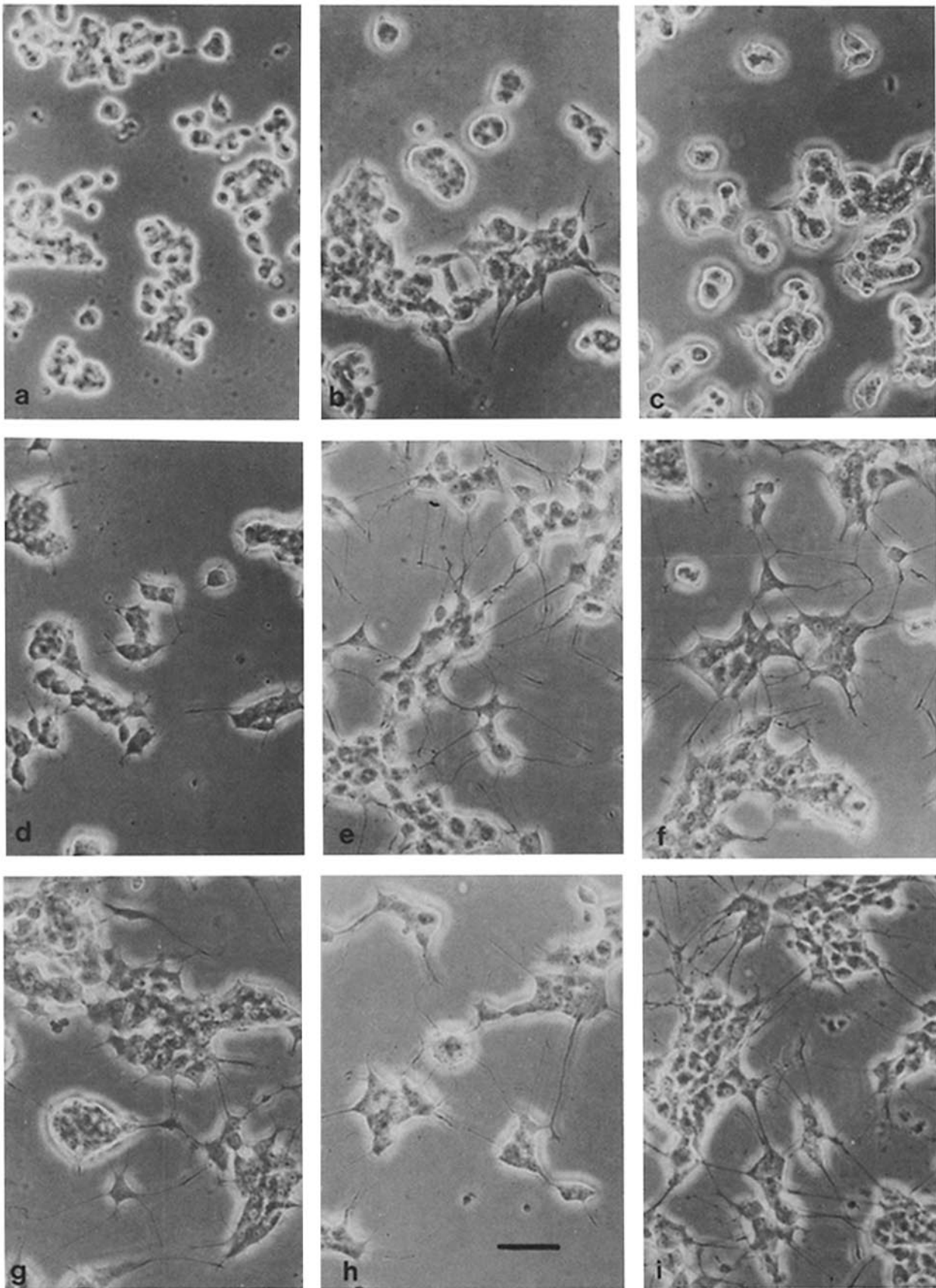


Figure 2. Effects of cAMP analogues on NGF-induced neurite outgrowth. PC12 cells were exposed to NGF (50 ng/ml) and cAMP derivatives (10^{-3} M) for 48 h. (a) Untreated control; (b) Bt₂cAMP; (c) 2'-O-BtcAMP; (d) NGF; (e) NGF plus Bt₂cAMP; (f) NGF plus 2'-O-BtcAMP; (g) NGF plus 6-ClcAMP; (h) NGF plus 8-BrcAMP; (i) NGF plus 8-PCTP. Bar, 50 μ m.

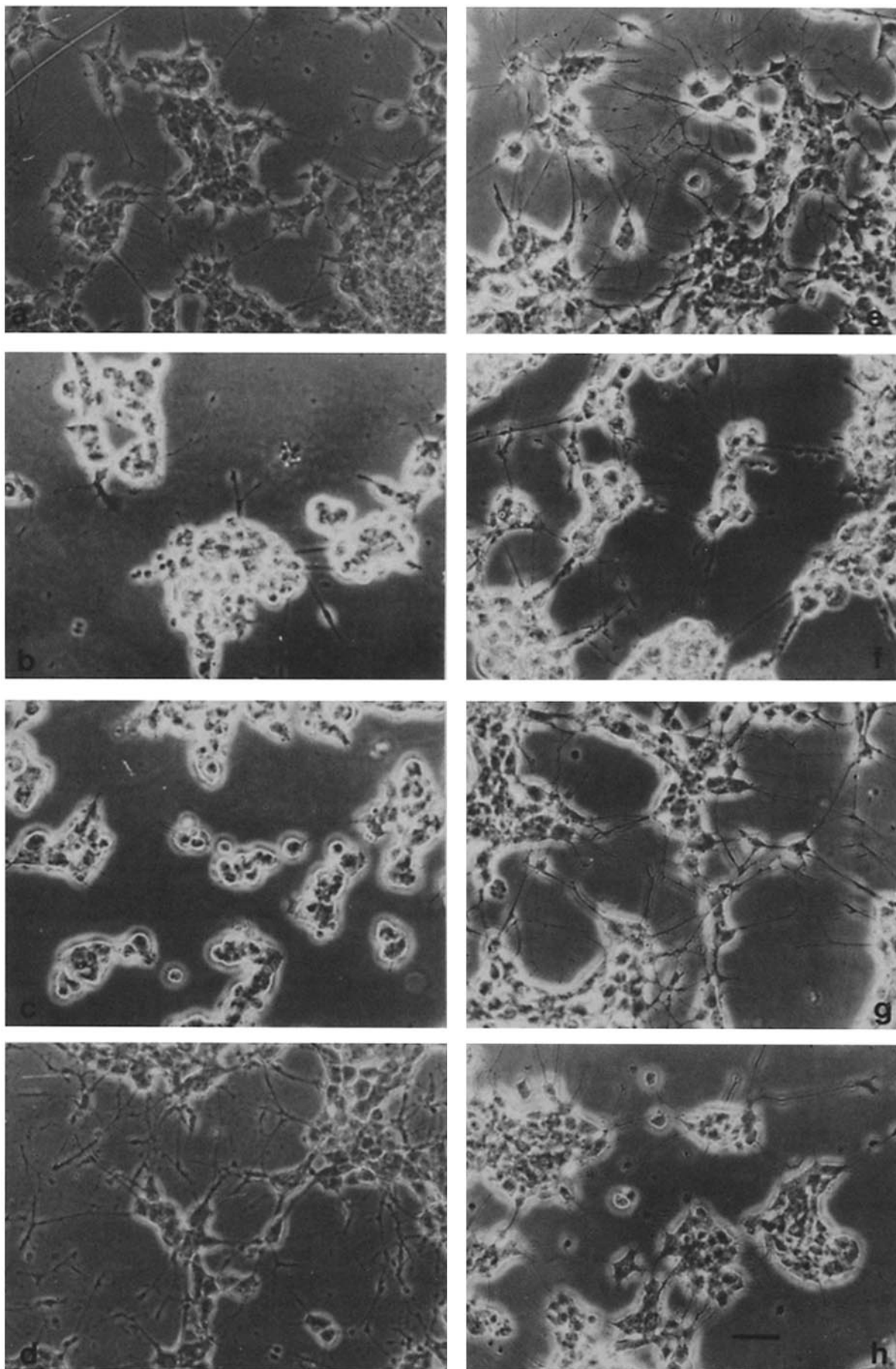


Figure 3. Long term effect of cAMP analogues on neurite outgrowth. Cells were exposed to NGF and cAMP analogues (10^{-3} M) for 6 d. (a) NGF alone; (b) Bt₂cAMP alone; (c) 6-ClcAMP alone; (d) NGF plus 8-PCTP; (e) NGF plus 2'-0-BtcAMP; (f) NGF plus Bt₂cAMP; (g) NGF plus 6-ClcAMP; (h) NGF plus 8-BrcAMP. Bar, 50 μ m.

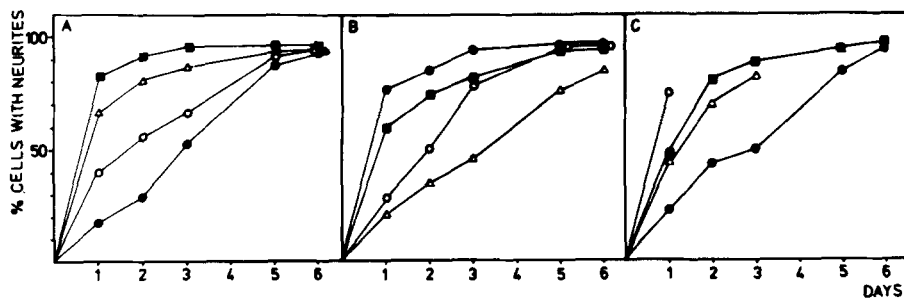


Figure 4. Time courses of appearance of neurites. PC12 cells were exposed to NGF (50 ng/ml) and cAMP analogues (10^{-3} M) simultaneously; forskolin was added at a concentration of 10^{-5} M. (A) ●, NGF; ○, 8-BrcAMP; △, cAMP; ■, Bt₂cAMP; (B) ●, Forskolin; ■, 2'-O-monobutyl cAMP; ○, 6-ClcAMP; △, 8-BrcGMP; (C) ○, GAB; ■, (Sp)-cAMPS; △, 8-PCTP; ●, (Rp)-cAMPS. Data represent the mean of triplicate experiments.

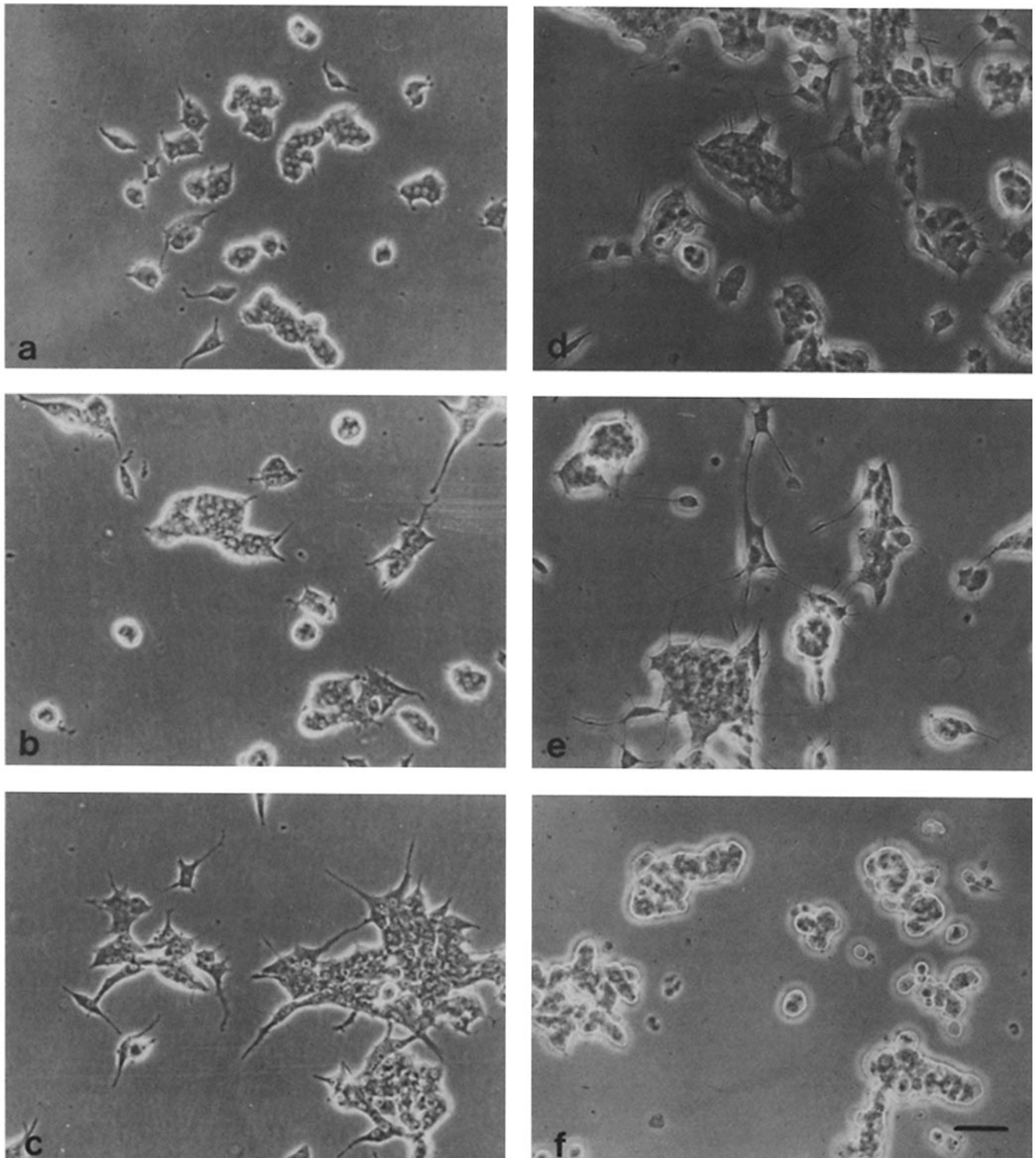


Figure 5. Effect of the cAMP antagonist (Rp)-cAMPS on NGF-mediated neurite outgrowth. Phase micrographs are shown of PC12 cells incubated for 1 d (a-c) or 3 d (d-f) with (a and d) NGF alone or (f) (Rp)-cAMPS (10^{-3} M) alone; and in the simultaneous presence of NGF and (b and e) (Rp)-cAMPS (10^{-3} M) or (c) (Sp)-cAMPS (10^{-3} M). Bar, 50 μ m.

processes, in contrast to 25% neurite-bearing cells in NGF-treated cultures (Fig. 4A). cAMP alone did not produce neurites, yet in combined action with NGF not only increased neurite formation but also had a network stabilizing effect as observed in cultures treated for up to 6–8 d (not shown). Dose-response evaluation revealed a maximal effect of cAMP at a concentration of 10^{-4} M, determined after 24 and 48 h of treatment.

Effect of (Rp)-cAMPS

The simultaneous addition of the cAMP antagonist (Rp)-cAMPS (10^{-3} M) and NGF to the growth medium of PC12 cells had no stimulatory or inhibitory action on NGF-induced process formation (Figs. 4C and 5). Also, preincubation of PC12 cells in the presence of 10^{-3} M (Rp)-cAMPS for up to 3 d did not affect subsequent NGF-induced neurite outgrowth in the continued presence of (Rp)-cAMPS (data not shown). Thus, the cAMP antagonist (Rp)-cAMPS could not inhibit the action of NGF on the initiation of neurite outgrowth. However, when (Rp)-cAMPS was added to cultures containing the cAMP agonist (Sp)-cAMPS and NGF, the potentiation of neurite outgrowth caused by the (Sp)-cAMPS diastereomer was markedly inhibited (Fig. 6). Quantitative evaluation indicated that the number of processes of PC12 cells after 3 d in NGF and (Sp)-cAMPS (10^{-4} M) was decreased in the presence of (Rp)-cAMPS at concentrations of 10^{-4} and 10^{-3} M (Fig. 6). Thus indeed, the (Rp)-cAMPS derivative inhibited a cAMP-specific effect in PC12 cells, which was caused by the exogenous addition of cAMP analogues to the culture medium. Cellular viability was determined by exclusion of Trypan blue. In all cases cell death, after 72 h of incubation time did not exceed 7–10%.

Uptake of (Sp)- and (Rp)-cAMPS by PC12 Cells

The hydrophobicity of cAMP analogues has been extensively studied before and the (Sp)- and (Rp)-cAMPS diastereomers have been described to have similar physico-chemical characteristics (2). When (Sp)-cAMPS was added to the culture medium of PC12 cells at a concentration of 10^{-3} M and aliquots of the cellular supernatant were analyzed by HPLC at various times, we found that after 72 h of incubation 23% of the exogenously applied (Sp)-cAMPS had disappeared from the medium (Fig. 7). Since PC12 cells do not excrete phosphodiesterase activity and the (Sp) isomer is stable in serum containing medium as well as in culture medium conditioned by PC12 cells for 72 h (Richter-Landsberg, C., and Th. Braumann, manuscript in preparation), the metabolite found in the medium must have its origin within the cells. The time

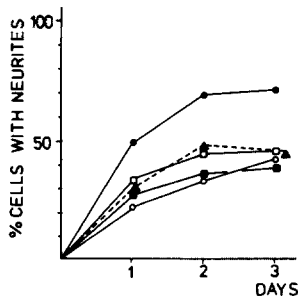


Figure 6. Inhibitory effect of (Rp)-cAMPS on neurite outgrowth in PC12 cells stimulated by the simultaneous presence of NGF and (Sp)-cAMPS. The percentage of neurite bearing clumps was analyzed in cultures exposed to NGF (○); 10^{-3} M (Rp)-cAMPS plus NGF (■), 10^{-4} M (Sp)-cAMPS plus NGF (●); NGF plus 10^{-4} M (Sp)-cAMPS

simultaneously with 10^{-4} M (Rp)-cAMPS (▲); and 10^{-3} M (□), respectively. Values are expressed as mean of a triplicate experiment.

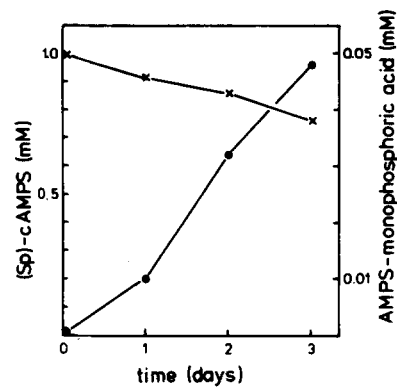


Figure 7. Uptake of (Sp)-cAMPS and release of its metabolite AMPS-monophosphoric acid. (Sp)-cAMPS (1 mM) was added at day 0, and the cell supernatant was analyzed by HPLC at the indicated times. After 72 h of incubation time the concentration of (Sp)-cAMPS (×) had reached a level of 0.77 mM, and the intracellularly formed metabolite AMPS-monophosphoric acid (●) was detectable in the cell supernatant.

course of the uptake of (Rp)-cAMPS revealed identical results (not shown). However, no metabolite was found in the culture medium even after 72 h of incubation, sustaining previous findings, that this derivative is extremely stable against phosphodiesterase activity (Braumann, Th., and B Jastorff, manuscript in preparation). We could show by HPLC analysis that 3% of the (Rp)-cAMPS, which had entered the cells was extractable by sonication, whereas the rest remained tightly bound to the cellular pellet. Thus, the phosphorothioates indeed penetrate PC12 cellular membranes and therefore exert their differential action on cAMP-dependent protein kinases within PC12 cells.

Preliminary experiments indicate that in PC12 cells the (Rp)-cAMPS derivative has an inhibitory effect on the cAMP-dependent phosphorylation of a 200-kD protein. This protein was found to be stimulated by Bt_2cAMP and (Sp)-cAMPS at 10^{-3} M after 24 h of incubation (Richter-Landsberg, C., unpublished observations).

Effect of Forskolin

The adenylate cyclase activator forskolin has been described to stimulate adenylate cyclase in PC12 cells and raise the intracellular cAMP level by a factor of 50 at a concentration of 10^{-6} M (26, 27). We have analyzed the effect of forskolin on neurite formation at concentrations from 10^{-10} to 10^{-4} M. Forskolin (10^{-7} – 10^{-4} M) alone, like Bt_2cAMP , caused no formation of a neuritic network, but initiated 20–30% of the cells to extend short non-interlinking processes and to form large aggregates, as observed in cultures treated for up to 6–8 d (Fig. 8c). Exposure of PC12 cells to both NGF and forskolin produced a stimulation of process formation by far exceeding the effect of NGF or forskolin alone. After 24 h already 70–80% of the cells had long interconnected neurites (Fig. 8a) and a neural network was formed within 3 d (Fig. 8b) and continuously elaborated after prolonged treatment with both agents (Fig. 8, d and f). Fig. 9 shows that a half maximal effect of forskolin occurs at 2×10^{-8} M and a maximal effect occurs at 10^{-6} M. The combined action of NGF and forskolin seemed to maintain and stabilized the neuronal network elaborated by PC12 cells even better than NGF alone (Fig.

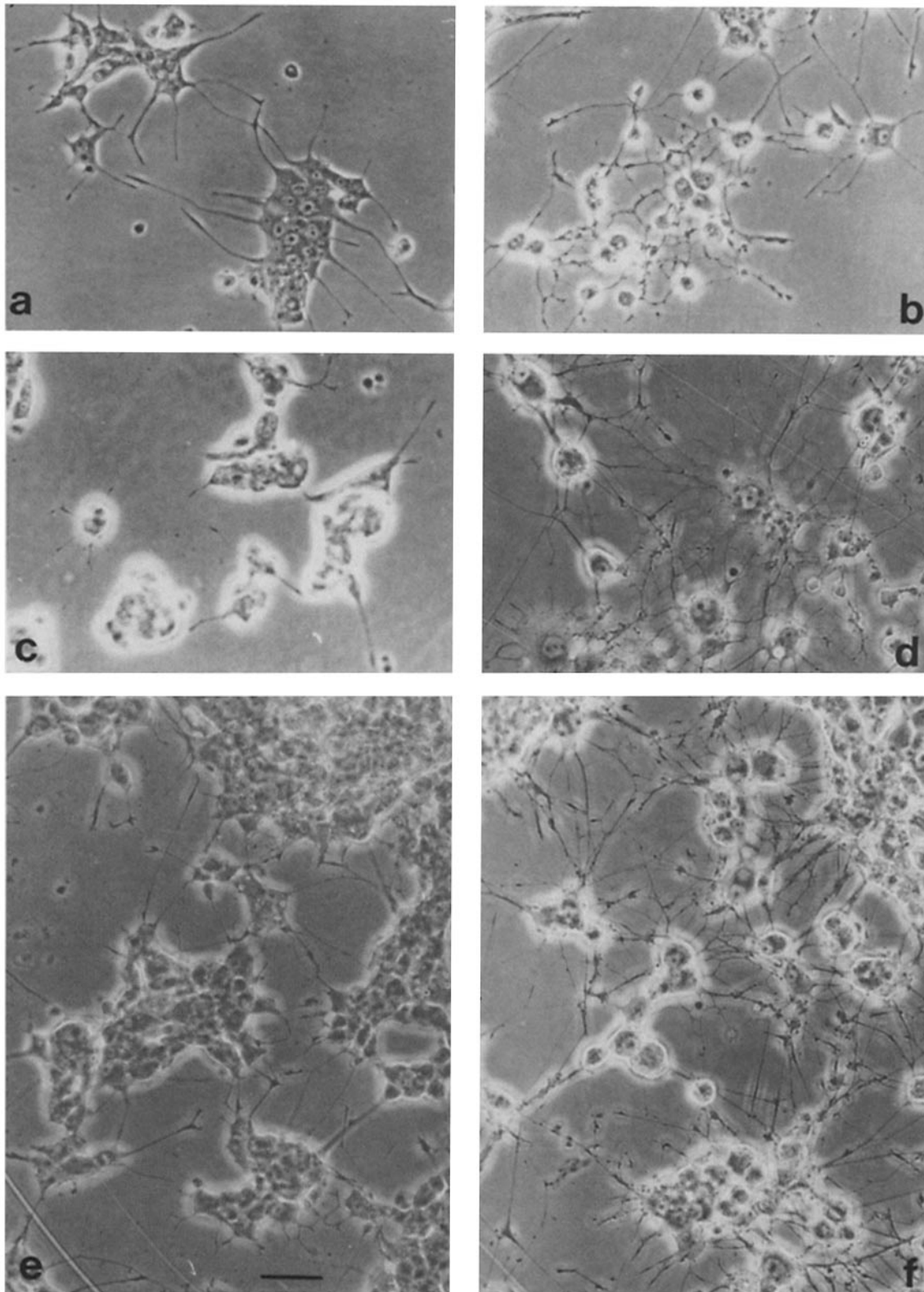


Figure 8. Potentiation of NGF-induced neurite outgrowth by forskolin. Phase micrographs are shown of PC12 cells exposed to (*a*, *b*, and *d*) NGF plus forskolin (10^{-5} M) for 1, 3, and 6 d, respectively. In *c*, cells were treated with forskolin alone (10^{-5} M) for 6 d. *e* and *f* depict micrographs of cells treated with NGF (*e*) and with NGF plus forskolin (10^{-6} M) (*f*) for 6 d. Bar, 50 μ m.

2*f*). It is interesting to note that after incubation with high concentrations of forskolin (10^{-4} – 10^{-5} M) PC12 cell volume appeared to decrease and a shrinkage of the cells was observed. At this high concentration long term neurite extension (>6 d)

was inhibited and many of the neurites failed to establish growth cone-like structures. In contrast, at lower concentrations of forskolin ($<10^{-6}$ M), cells seemed to rather increase their volume. Cultures treated with forskolin contained many

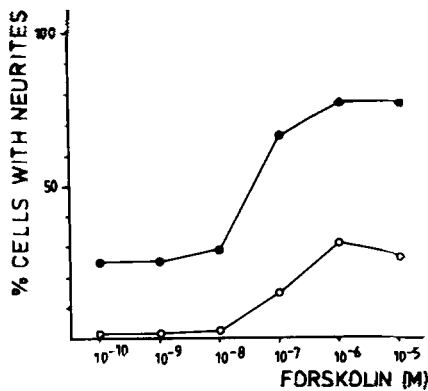


Figure 9. Dose response relationship of forskolin on NGF-induced neurite outgrowth in PC12 cells. Cells were incubated with increasing concentrations of forskolin (○), or forskolin plus NGF (●) for 24 h. The value for NGF alone at this time point was 23%. Numbers represent mean percentage of triplicate experiments.

giant cells with long branched neurites, as has been observed also for 8-BrcAMP.

Discussion

The aim of this study was to investigate further the role of cAMP on NGF-mediated neurite outgrowth in PC12 cells. The data indicate that a specific cAMP effect is observed during the treatment of PC12 cells with cAMP, its analogues or the potent adenylate cyclase activator forskolin. However, cAMP does not act as a second messenger for NGF-induced neurite outgrowth, as has been discussed (6, 15, 32, 33). This conclusion is based on the following evidence: (a) Neither forskolin nor cAMP or its analogues induced neurite outgrowth to the same extent as NGF. (b) The simultaneous addition of NGF and cAMP analogues or forskolin potentiated neurite outgrowth in PC12 cells synergistically, thus indicating that they act through differential mechanisms. (c) The cAMP antagonist (Rp)-cAMPS did not exert inhibitory actions on NGF-induced process formation in PC12 cells. (d) The synergistic potentiation of neurite outgrowth produced by the simultaneous addition of NGF and the cAMP agonist (Sp)-cAMPS was successfully inhibited by the addition of (Rp)-cAMPS, and neurite outgrowth was repressed almost to the level normally seen with NGF alone. The (Rp)-cAMPS analogue neither alone nor in combination with NGF exerts any morphological changes or synergistic effects in PC12 cells. (Rp)-cAMPS has previously been described to compete with cAMP for the binding to the regulatory subunit of protein kinases (4, 24). Upon binding, the release of the catalytic subunit is inhibited, and therefore the activation of cAMP-dependent protein kinases is blocked (4). This has been demonstrated for protein kinases of rat hepatocytes (30, 31). (Rp)-cAMPS has also been shown to inhibit cAMP-induced *in vitro* phosphorylation of microtubule-associated protein 2 (MAP2) in microtubules prepared from beef brain (28). NGF-induced neurite formation was not inhibited by this derivative. This indicates that cAMP activation of protein kinases is neither sufficient nor necessary for NGF-induced neurite outgrowth in PC12 cells. It is, however, conceivable that the constant application of agents that increase the intracellular levels of cAMP indirectly trigger NGF-like responses.

We have found that cAMP itself added to the culture

medium stimulates NGF-induced neurite outgrowth, although the polarity of the molecule and metabolizing enzymatic activities supposedly impede its passage through the cellular membrane (25). In recent years, cAMP receptors have been identified in mammalian brain in cytosol as well as membrane fractions (37). In neuroblastoma-glioma hybrid cells Walter et al. (36) have identified free type I regulatory subunit and demonstrated its regulation by Bt₂cAMP. The possibility was discussed that cAMP receptor proteins have additional functions apart from inhibiting the catalytic subunit of protein kinases and may regulate cellular functions such as growth and differentiation (36). One might further hypothesize that cAMP acts as a regulator of differentiation of nerve cells. Sympathetic neurons and adrenal medullary cells originate from the neural crest and thus have common precursors. PC12 cells under normal growth conditions are considered to represent the neoplastic counterpart of adrenal chromaffin cells, and in the presence of NGF they resemble sympathetic neurons (9). cAMP has been described as having differential effects on NGF-induced neurite outgrowth from adrenal medullary chromaffin cells, sympathetic neurons, and PC12 cells (39). These authors showed that Bt₂cAMP, theophylline, or cholera toxin in combination with NGF enhanced neurite outgrowth in PC12 cells and sympathetic neurons, whereas process formation from chromaffin cells under the same conditions was suppressed (39). Thus, the combined presence of NGF and cAMP may influence the developmental expression of neural crest derived cells to either become the neuronal or adrenal phenotype.

Two major effects of NGF have been discussed: first, a slow and transcription-dependent process, providing the synthesis of proteins necessary for the formation and growth of neurites, and second, a fast and transcription-independent process. This fast process may regulate the state of assembly and the activity and organization of these yet unidentified proteins and thereby promote neurite outgrowth (7, 10). The latter process might be related to the action of cAMP. Neurite initiation by NGF from PC12 cells requires RNA synthesis, whereas neurite regeneration of "primed" cells is a transcription-independent process (3). PC12 cells cannot be primed by Bt₂cAMP, yet the simultaneous addition of Bt₂cAMP and NGF enables the cells to initiate neurite outgrowth by a RNA-synthesis independent mechanism (13, 14, 16). Thus, primed cells as well as cells treated with NGF and Bt₂cAMP can respond rapidly to NGF for regenerative or plastic processes.

We have found that although after 6–8 d of treatment with NGF alone or in the presence of NGF plus cAMP analogues or forskolin, the maximal percentage of neurite-bearing cells was reached under both conditions; the neuronal network, however, was better maintained and more extensively branched in the presence of the combined treatment. Hence, cAMP was not necessary for the initiation of neurite outgrowth but helped to stabilize and maintain established connections.

cAMP or its analogues exerted their morphological effects at relatively high, unphysiological concentrations, requiring the continued presence of the agents. It has to be taken into consideration that cAMP metabolites may be produced during the long incubation and may induce specific physiological events during the differentiation of PC12 cells. These questions are currently under investigation in our laboratory.

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