cAMP analogs promote survival and neurite outgrowth in cultures of rat sympathetic and sensory neurons independently of nerve growth factor

(neurotrophic agents/neuronal regeneration/neuronal differentiation/8-(4-chlorophenylthio)-cAMP/8-bromo-cAMP)

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ABSTRACT Nerve growth factor (NGF) is a neurotrophic agent for sympathetic and embryonic sensory neurons both *in vivo* and *in vitro*. We report here that the membrane-permeant cAMP analogs, 8-(4-chlorophenylthio)-cAMP and 8-bromo-cAMP, can replace NGF in promoting long-term survival and neurite outgrowth in cultures of rat neonatal sympathetic and embryonic sensory neurons. N^6-substituted analogs, including the more commonly used $N^6,O^{2'}$-dibutyryl-cAMP, are less efficacious. Additivity and switching experiments indicate that the cAMP analogs affect the same neuronal population as that maintained by NGF. However, unlike NGF, the cAMP analogs do not evoke somatic hypertrophy. Moreover, studies with sympathetic neurons reveal that the neurotrophic actions of the cAMP analogs, but not of NGF, are blocked by the axial diastereoisomer of adenosine 3',5'-phosphorothioate, a competitive cAMP antagonist. Thus, the mechanism by which cAMP analogs promote neuronal survival and differentiation appears to involve activation of cAMP-dependent protein kinases, whereas, in contrast, the same effects of NGF neither require nor are mediated by such a pathway. Furthermore, the different efficacies observed with N^6- and C8-substituted cAMP analogs suggest that this neurotrophic pathway may involve differential activation of the regulatory subunits of cAMP-dependent protein kinases. The presence of this parallel, cAMP-responsive, neurotrophic pathway in at least two types of NGF-responsive neurons may be developmentally important and has the potential to be exploited for the treatment of injuries or diseases affecting these and possibly other nerve cells.

A wide variety of extracellular signals, both inside and outside the nervous system, produce many of their diverse responses by regulating the phosphorylation of specific proteins. In particular, protein phosphorylation has been implicated as a molecular mechanism by which extracellular signals regulate various intracellular functions in nervous tissue, including neuronal excitability, neurotransmitter biosynthesis and release, and also neuronal growth, differentiation, and morphology (reviewed in ref. 1). One mechanism by which many extracellular signals regulate protein phosphorylation involves the synthesis of cAMP and subsequent activation of its target enzymes, the cAMP-dependent protein kinases (reviewed in ref. 2). In vertebrate nervous tissue, the regulatory subunits of cAMP-dependent protein kinases appear to be the major, if not the only, proteins that bind and are activated by cAMP (3, 4).

One of the best characterized mediators of nervous system development is the trophic polypeptide, nerve growth factor (NGF). NGF profoundly influences the differentiated state and maintenance of a variety of nerve cells, including sensory, sympathetic, and certain central nervous system neurons (5–8). In particular, previous studies have shown the inability of sympathetic and embryonic sensory neurons to survive *in vivo* (5, 9, 10) or *in vitro* (11, 12) in the absence of NGF. Although current knowledge of the molecular events orchestrating NGF's neurotrophic properties is limited, both transcription-independent and transcription-dependent pathways appear to be involved in NGF-promoted neurite outgrowth and neuronal differentiation (reviewed in ref. 13). In this regard, various groups (14–20) have reported that cAMP analogs and NGF have both synergistic and differential actions on NGF-responsive cells. cAMP analogs have been reported to modulate the phosphorylation of a similar group of proteins, as does NGF (14, 15). However, the effects of cAMP analogs and NGF on rat PC12 cell surface morphology (16) and neurite outgrowth from both PC12 cells (17–19) and embryonic chicken sensory ganglia (20) suggest divergent pathways of action.

To more fully understand the role of cAMP and phosphorylation in the mechanism of NGF's action, we exposed cultures of dissociated rat neonatal superior cervical ganglia and embryonic dorsal root ganglia to a variety of agents, including those that either mimic or elevate intracellular levels of cAMP. We report here that certain, but not all, permeant cAMP analogs can maintain survival of (and promote neurite outgrowth by) the same neuronal population as that maintained by NGF. Moreover, use of a competitive cAMP antagonist (19, 21–25) indicates that the actions of these analogs require activation of cAMP-dependent protein kinase(s), whereas, in contrast, NGF-promoted survival and neurite outgrowth neither require nor are mediated by way of cAMP.

A portion of these studies has previously appeared in abstract form (26).

MATERIALS AND METHODS

Tissue Culture. Postnatal day 1–3 rat superior cervical ganglion cells were dissociated and plated at a density of 1 ganglion per 16-mm or 35-mm culture dish as previously described (27). Embryonic day 15 rat dorsal root ganglion cells were dissociated and plated at a density of 8–11 ganglia per 35-mm culture dish as described by Salzer *et al.* (28), except that 15% fetal bovine serum was used in place of

Abbreviations: CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; 8Br-cAMP, 8-bromo-cAMP; Bt$_2$cAMP, $N^6,O^{2'}$-dibutyryl-cAMP; Oco$_2$cAMP, $N^6,O^{2'}$-dioctanoyl-cAMP; NGF, nerve growth factor; S_P-, R_P-cAMP[S], equatorial and axial diastereoisomers of adenosine 3',5'-phosphorothioate, respectively.
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human placental serum and chicken embryo extract. For most experiments, a laminin substrate (29) was utilized except where noted, when cultures were maintained on a collagen substrate (27). Cultures were treated with either NGF (50 ng/ml) or the agent to be tested, and the culture medium was changed every other day. Cultures highly enriched for neuronal cells were obtained by treatment with 10 μM cytosine arabinoside from the first to seventh day after plating (superior cervical ganglia cultures) or by continuous exposure to 10 μM fluorodeoxyuridine and 10 μM uridine (dorsal root ganglia cultures).

Determination of Neuronal Survival. For experiments utilizing 35-mm culture dishes, the number of neurons (phase-bright, neurite-bearing cells) was quantified in living cultures by counting under a phase-contrast microscope ($\times 200$). For each culture, at least two 1.0×23.0 mm diametrical strips (each representing 1.2% of the culture surface) were surveyed, and the neuronal number per strip was then used to calculate the total number of neurons per culture. For experiments utilizing 16-mm culture dishes, survival was quantified by fixing the cells in phosphate-buffered 2.5% glutaraldehyde and counting all neurons present.

Materials. Mouse 2.5S NGF was prepared from adult male submandibular glands as described by Mobley *et al.* (30). Tissue culture media were from GIBCO, and sera were from KC Biological (Lenexa, KS). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP), 8-bromo-cAMP (8Br-cAMP), 8-bromo-cGMP, and $N^6,O^{2'}$-dibutyryl-cAMP (Bt$_2$cAMP) were purchased from Boehringer Mannheim. Forskolin and $N^6,O^{2'}$-dioctanoyl-cAMP (Oco$_2$cAMP) were purchased from Calbiochem. Laminin was purchased from E-Y Laboratories (San Mateo, CA). The equatorial (S_P) and axial (R_P) diastereoisomers of adenosine $3',5'$-phosphorothioate (cAMP[S]) were generously supplied by B. Jastorff (University of Bremen, Bremen, F.R.G.). Bovine basic fibroblast growth factor was generously supplied by D. Gospodarowicz (University of California Medical Center, San Francisco). All other reagents were purchased from Sigma.

RESULTS

Effects of Various Agents on the Survival of Neonatal Rat Sympathetic Neurons. Cultures of dissociated neonatal rat superior cervical ganglion cells were exposed to a variety of agents, including those that either mimic or elevate intracellular levels of cAMP (Fig. 1). Two of the agents tested, CPT-cAMP and 8Br-cAMP, maintained neuronal survival and promoted neurite outgrowth to a similar extent as NGF (Figs. 1A and 2) for at least 1 month (Fig. 2H). These effects of CPT-cAMP and 8Br-cAMP were dose-dependent and had maximal activities between 100 and 300 μM and 0.3 to 1.0 mM, respectively (Fig. 1B). Furthermore, treatment with 10 μM cytosine arabinoside to essentially eliminate the nonneuronal cells (mainly Schwann cells and fibroblasts) did not alter the neurotrophic effects of the cAMP analogs. This indicates that these analogs interact directly with the neuronal population and do not mediate their neurotrophic actions by way of a secondary mechanism involving nonneuronal cells.

Fig. 1 also reveals that not all membrane-permeant cAMP analogs possessed maximal neurotrophic activity. Bt$_2$cAMP had less efficacy than NGF for promoting neuronal survival. Similar results were obtained with Oco$_2$cAMP. In addition, forskolin, an activator of adenylate cyclase in many cell types (31), was also without maximal neurotrophic activity. However, forskolin did potentiate the survival effects of a submaximal dose of CPT-cAMP; exposure of cultures to 100 μM CPT-cAMP in the presence of 30 μM forskolin resulted in maximal neuronal survival (not shown). The relative ineffectiveness of the N^6-substituted analogs and forskolin
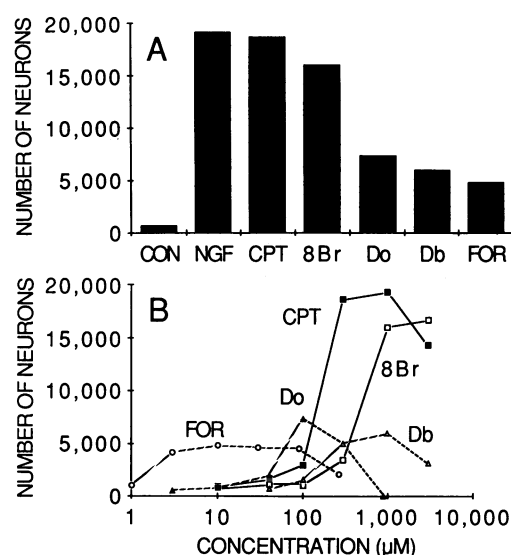

FIG. 1. Effects of various cAMP analogs and forskolin on the survival of neonatal rat sympathetic neurons after 8 days in culture. Highly enriched cultures of postnatal day 1 neurons were established. (*A*) Neuronal survival in the presence or absence (CON) of various agents. The data presented are for optimal survival doses as determined from dose–response experiments and were 50 ng of NGF per ml, 300 μM CPT-cAMP (CPT), 1.0 mM 8Br-cAMP (8Br), 10 μM forskolin (FOR), 100 μM Oco$_2$cAMP (Do), and 1.0 mM Bt$_2$cAMP (Db). (*B*) Dose–response relationships for the survival-promoting effects of CPT-cAMP (-■-), 8Br-cAMP (-□-), Oco$_2$cAMP (--▲--), Bt$_2$cAMP (--△--), and forskolin (--○--). Comparable dose–response curves were obtained in two additional, independent experiments.

was not due to general cytotoxic properties, since they did not interfere with (nor did they enhance) NGF-promoted neuronal survival or neurite outgrowth (not shown).

The survival requirement of rat sympathetic neurons appears to be highly selective for cAMP analogs and NGF. Treatment with 8-bromo- or dibutyryl-cGMP (0.01–2 mM), basic fibroblast growth factor (300 pM), insulin (200 nM), K^+ (40 mM), or phorbol 12-myristate 13-acetate (4 nM–1 μM), an activator of protein kinase C, failed to maintain neuronal survival (not shown). In addition, these agents had no evident effect on NGF-promoted survival or neurite outgrowth (not shown). These concentrations of insulin, K^+, and phorbol 12-myristate 13-acetate have been reported to promote survival and neurite outgrowth in cultures of chicken sympathetic neurons (32, 33).

cAMP Analogs and NGF Affect the Same Population of Rat Sympathetic Neurons. Since the recovery of neurons in our culture system is less than the number initially plated (57–67% after 7 days), experiments were performed to determine if the same neuronal population responds both to NGF and to cAMP analogs. In one set of studies, 7-day-old cultures initiated in the presence of NGF were rinsed extensively and then were maintained for an additional 4 days in the presence of anti-NGF antiserum or anti-NGF antiserum plus CPT-cAMP. Removal of NGF resulted in the total loss of neuronal viability (Fig. 2A), whereas removal of NGF coupled with the addition of CPT-cAMP afforded maintenance of neuronal survival and neurite integrity (Fig. 2B).

In another set of experiments, sympathetic neuron cultures were initiated in the presence of either NGF, CPT-cAMP, or CPT-cAMP plus NGF. After 7 days, the cultures were extensively rinsed and then maintained for an additional week in the presence of either NGF, CPT-cAMP plus anti-NGF antiserum, CPT-cAMP plus NGF, or anti-NGF antiserum. The numbers of surviving neurons were determined at various times throughout the experiment (Fig. 3).
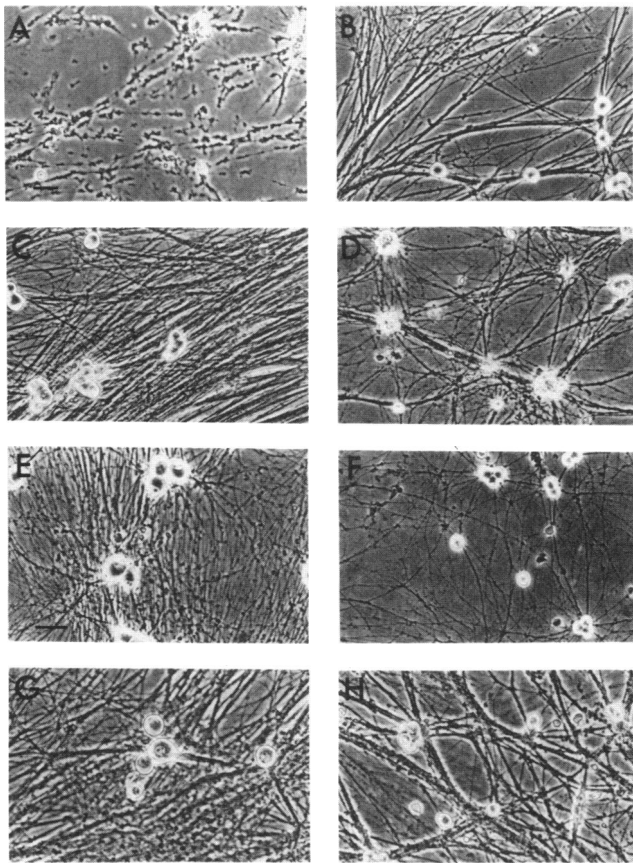
FIG. 2. Highly enriched cultures of postnatal day 2 rat sympathetic neurons. (*A* and *B*) Neurons were maintained on a collagen substrate (27) for 4 days in the presence of 50 ng of NGF per ml, washed extensively with NGF-free medium, and cultured for 4 additional days in the presence of anti-NGF antiserum without (*A*) or with (*B*) 500 μM CPT-cAMP. (*C* and *D*) Neurons were cultured for 8 days on a collagen substrate in the presence of 50 ng of NGF per ml (*C*) or 500 μM CPT-cAMP (*D*). (*E* and *F*) Neurons were cultured for 8 days on a laminin substrate (29) in the presence of 50 ng of NGF per ml (*E*) or 500 μM CPT-cAMP (*F*). (*G* and *H*) Neurons were cultured for 1 month on a collagen substrate in the presence of 50 ng of NGF per ml (*G*) or 500 μM CPT-cAMP (*H*). Comparable results were achieved when 1 mM 8Br-cAMP was used instead of CPT-cAMP. (Bar = 50 μm.)
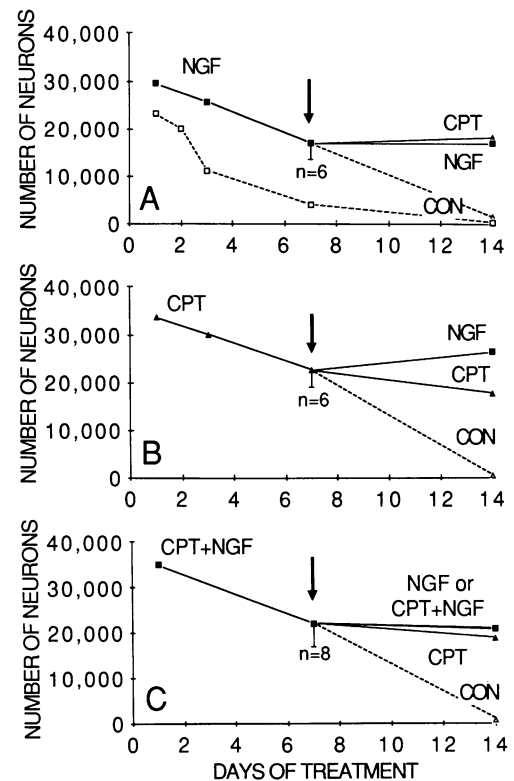

FIG. 3. NGF and CPT-cAMP maintain neuronal survival of identical populations of sympathetic neurons. Highly enriched cultures of postnatal day 1 neurons were maintained on a laminin substrate in the presence of 50 ng of NGF per ml (*A*), 500 μM CPT-cAMP (*B*), or CPT-cAMP plus NGF (*C*) for 7 days. The neurons were rinsed extensively with NGF-free medium on day 7 (arrow) and then maintained for an additional 7 days in the presence of NGF (■ in *A* and *B*), CPT-cAMP plus anti-NGF antiserum (CPT; ▲), CPT-cAMP plus NGF (CPT + NGF; ■ in *C*), or anti-NGF antiserum (CON, △). Cultures were also maintained without additives (--□-- in *A*). Neuronal numbers were determined as in Fig. 1. The values after 14 days are the average of counts from duplicate dishes; variation between the duplicates was 5–10%. Comparable results were obtained in four additional, independent experiments.

First, these results show that the numbers of neurons surviving after 1 and 7 days are not significantly greater in the presence of CPT-cAMP plus NGF than they are in the presence of either agent alone. Second, switching from one treatment to the other did not result in significant loss of neuronal viability. Thus, NGF maintained the neurons initially maintained with CPT-cAMP, just as CPT-cAMP maintained those neurons initially maintained by NGF. This demonstrates that the same sympathetic neurons respond both to NGF and to CPT-cAMP. Third, the response of neurons to CPT-cAMP in the presence of anti-NGF antiserum indicates that the action of this drug is not indirectly due to stimulation of NGF synthesis and release.

cAMP Analogs and NGF Promote Different Morphological Responses by Sympathetic Neurons. Although CPT-cAMP and 8Br-cAMP share with NGF the ability to maintain and promote neurite outgrowth by cultured sympathetic neurons, the responses they generate differ in several respects. In particular, when sympathetic neurons were plated on a collagen-coated substrate in the presence of NGF, they produced neurites within 1 day. In contrast, the appearance of neurites on a collagen substrate was delayed by 1–2 days when plating was in the presence of cAMP analogs. Under

these conditions, neurites elicited by cAMP analogs were much more highly fasciculated and appeared to be less extensive than those elicited by NGF (Fig. 2 *C* and *D*). However, when the neurons were plated on a laminin substrate, the rate of neurite formation and the extent of fasciculation were similar for both types of treatment; nevertheless, the density of outgrowth was consistently less in the presence of cAMP analogs (Fig. 2 *E* and *F*). Furthermore, relative to their state at the time of plating, NGF produced a marked hypertrophy of neuronal cell bodies in cultures utilizing either substrate (Fig. 2 *C*, *E*, and *G*); in contrast, this never occurred with 8Br-cAMP or CPT-cAMP, even in cultures maintained for one month (compare Fig. 2 *D*, *F*, and *H*). However, addition of NGF to cultures maintained with cAMP analogs did produce somatic hypertrophy as well as enhancement in neurite density (not shown).

The Neurotrophic Activities of cAMP Analogs and NGF in Sympathetic Neurons Occur by Way of Independent Pathways. To determine whether the neurotrophic effects of cAMP analogs and NGF are mediated by way of the same primary pathway, we employed S_P- and R_P-cAMP[S]. These analogs differentially affect cAMP-dependent protein kinases (21–23). S_P-cAMP[S] activates cAMP-dependent protein kinases in the same manner as cAMP, whereas R_P-cAMP[S] binds to the regulatory subunits but does not

dissociate the holoenzyme, thereby behaving as a competitive cAMP antagonist (19, 21–25). When assessed after 4 days of treatment, R_P-cAMP[S] had little or no effect on survival or neurite outgrowth in cultures initiated with NGF, but it almost completely blocked the survival-promoting effects of CPT-cAMP (Fig. 4). S_P-cAMP[S] exerted a moderate effect on neuronal survival when added alone but did not alter the neurotrophic effects of CPT-cAMP or NGF (not shown). This indicates that the mechanism by which cAMP analogs maintain neuronal survival involves activation of cAMP-dependent protein kinases and that, in contrast, NGF-promoted survival and neurite outgrowth by sympathetic neurons neither requires nor is mediated by such a pathway. These findings are consistent with others that appear to preclude a required role for cAMP in the NGF mechanism (16–18) and, in particular, with those of Richter-Landsberg and Jastorff (19), who reported that R_P-cAMP[S] blocks the morphologic actions of cAMP analogs, but not of NGF, on PC12 cells.

Effects of CPT-cAMP and NGF on Embryonic Rat Sensory Neurons. To determine if cAMP analogs can maintain rat sensory neurons, cultures of dissociated embryonic rat dorsal root ganglion cells were initiated in the presence of either NGF, CPT-cAMP, or NGF plus CPT-cAMP. The numbers of surviving neurons were determined after 24 or 48 hr (Table 1). As with sympathetic neurons, the numbers of sensory neurons surviving after 24 or 48 hr in culture were not significantly different in the presence of either NGF or CPT-cAMP. Furthermore, cotreatment with NGF plus CPT-cAMP did not enhance neuronal survival. This lack of additivity suggests that a similar population of sensory neurons can be maintained by either cAMP analogs or NGF. As observed in sympathetic neuron cultures, sensory neurons maintained with CPT-cAMP showed a lack of somatic hypertrophy and less extensive neurite outgrowth as compared to cultures maintained with NGF (not shown).

DISCUSSION

Our results demonstrate that certain cAMP analogs support the survival and differentiation of the same population of rat sympathetic neurons that responds to NGF. Furthermore, the finding that a competitive cAMP antagonist, R_P-cAMP[S], blocks these effects of cAMP analogs but not those of NGF demonstrates the involvement of distinct primary mechanisms. The presence of parallel neurotrophic pathways is further supported by the different morphological

Table 1. Effects of NGF and CPT-cAMP on survival of dissociated embryonic rat dorsal root ganglion neurons in culture

Treatment	Neuronal survival	
	Exp. 1*	Exp. 2†
None	13,427 ± 363 (3)	6,221; 10,049
NGF (50 ng/ml)	59,740 ± 2451 (3)	24,926; 39,716
CPT-cAMP (500 μM)	59,986 ± 1840 (4)	38,585; 50,765
NGF + CPT-cAMP		29,537; 40,934

Enriched cultures of dissociated embryonic day 15 rat sensory neurons were established, and neuronal survival was determined.
*Neuronal survival was determined after 24 hr. Results are means ± SEM; the numbers of replicate cultures are given in parentheses.
†Neuronal survival in an additional, independent experiment determined after 48 hr.

responses generated by the analogs and NGF. Unlike NGF, the cAMP analogs do not evoke somatic hypertrophy. In addition, neurite outgrowth on a collagen substrate is both delayed in its appearance and more highly fasciculated in the presence of cAMP analogs as compared to the outgrowth generated by NGF.

Our studies also demonstrate that CPT-cAMP promotes survival and neurite outgrowth by dissociated embryonic rat sensory neurons. These observations, coupled with reports that cAMP analogs affect cell survival (34) and neurite outgrowth (35, 36) in other neuronal populations, suggest that there may be a more general occurrence of cAMP-mediated neurotrophic pathways.

The use of a variety of cAMP analogs has revealed differential effects of C-8- and N^6-substituted cAMP derivatives. Thus, N^6-substituted analogs are less efficacious than C-8-substituted analogs in promoting sympathetic neuron survival. This is consistent with reports that the N^6-substituted analog Bt_2cAMP is relatively ineffective in certain other systems known to be regulated by cAMP (25, 37, 38). Conformation studies indicate that C-8-substituted analogs have a predominately *syn* configuration, whereas, in contrast, N^6-substituted analogs display an *anti* conformation (39, 40). These observations raise the possibility that the differential effects of the C-8- and N^6-substituted analogs could arise from selective activation of specific forms of cAMP-dependent protein kinase. In support of this, various analogs apparently display selectivity of interaction with the two types (41–43) [or isoforms thereof (44)] of cAMP-dependent protein kinase regulatory subunits R_I and R_{II}. In addition, C-8- and N^6-substituted analogs can display quite distinct specificities for interaction with the two intrachain cAMP binding sites found on R_I and R_{II} (45, 46). Moreover, several different agents that elevate intracellular cAMP levels have been reported to lead to selective activation of the R_I and R_{II} isotypes (47–49). Thus, if the neurotrophic actions of cAMP and its analogs do indeed require differential effects on the various forms of cAMP-dependent protein kinase, then the response of a given cell to an agent that elevates or mimics cAMP will be governed both by the types and distribution of cAMP-dependent protein kinase forms that it possesses and by the nature of the agent itself.

Whatever the source of differences in neurotrophic activity of the various analogs, since many previous studies on the role of cAMP in neuronal function have exclusively utilized Bt_2cAMP, the present results suggest that future work should also include C-8-substituted derivatives.

The presence of a cAMP-mediated neurotrophic pathway parallel to that of NGF raises several intriguing possibilities. For instance, the early differentiation of mammalian sympathetic neurons appears to occur independently of NGF (50, 51). Our observations thus raise the possibility that such development may be mediated by way of a cAMP-dependent pathway. In addition, it has been reported (52) that the
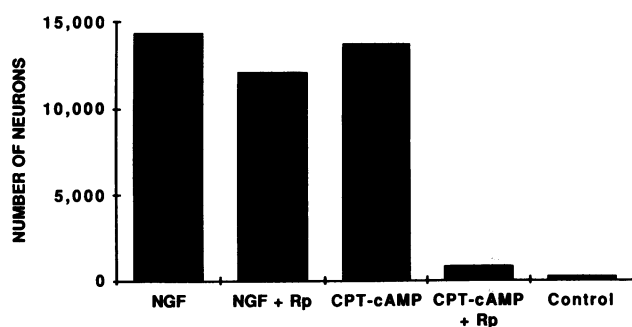

Fig. 4. Differential inhibition of the survival-promoting effects of NGF and CPT-cAMP. Postnatal day 3 rat sympathetic ganglion cells were dissociated and plated at 1 ganglion per 16-mm culture dish onto a collagen substrate in the presence of either 50 ng of NGF per ml, NGF plus 1.0 mM R_P-cAMP[S] (NGF + Rp), 500 μM CPT-cAMP, CPT-cAMP plus R_P-cAMP[S] (CPT-cAMP + Rp), or no additive (Control). After 4 days, all neurons present in each dish were counted. The values are the average of counts from duplicate dishes; variation between the duplicates was 5–10%. Comparable results were obtained in two additional, independent experiments.

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activity of the cAMP generating system in rat sympathetic neurons is directly proportional to neurite length. The establishment of extensive neuronal networks could thus account for the decreased NGF dependence that occurs with maturation of sympathetic neurons *in vivo* (53) and *in vitro* (54). Such a mechanism could also explain the capacity of sympathetic neurons in long-term cultures to undergo NGF-independent neurite regeneration (55). Finally, it is worth considering the possibility that neurotransmitters or peptides that elevate intracellular cAMP levels may exert neurotrophic actions on sympathetic or sensory neurons.

Our observations may also prove useful for illuminating the molecular events involved in neurotrophic factor action. A common property of NGF and cAMP analogs is the induction of rapid changes in the phosphorylation of specific cellular proteins (14, 15). This suggests that protein phosphorylation may play a causative role in both neuronal survival and neurite outgrowth and that comparison of the phosphorylations evoked by NGF and cAMP analogs will be relevant to understanding the similarities and differences in responses to these agents. Also, since the cAMP analogs discussed here permit otherwise NGF-requiring neurons to be cultured without the factor, it will be possible to examine the primary events that occur after exposure to NGF.

Finally, the present findings may have clinical relevance. By promoting survival, differentiation and/or regeneration, these analogs have potential use for treating injuries or diseases affecting mammalian sympathetic, sensory, and possibly other neurons.

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