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Cyclic AMP Prevents an Increase in GAP-43 but Promotes Neurite Growth in Cultured Adult Rat Dorsal Root Ganglion Neurons

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High expression of the growth-associated protein GAP-43 in neurons is correlated with developmental and regenerative axon growth. It has been postulated that during development and after injury, GAP-43 expression is elevated due to the unavailability of a target-derived repressive signal, but that GAP-43 expression then declines upon target contact. Here we examine the cyclic AMP second messenger signaling pathway to determine if it might mediate retrograde transmission of a signal which represses GAP-43 expression and inhibits growth. Cultures of adult rat dorsal root ganglia were chronically exposed to membrane-permeable analogs of cyclic AMP and activators of adenyl cyclase. These treatments caused GAP-43 protein levels to decrease in a dose-dependent manner, although neuronal survival was not affected. GAP-43 mRNA was also decreases by cyclic AMP. GAP-43 protein levels were not repressed by neurotrophins, cytokines, or other agents. Surprisingly, cyclic AMP caused an increase in the rate of neurite outgrowth, even though the neurons were partially depleted of GAP-43. Growth stimulation was quickly inducible and reversible, could occur in the presence of transcription inhibitors, and did not entail alterations in branching pattern. These findings suggest that axon growth involving high levels of GAP-43 is distinct from the growth stimulation which is rapidly induced by cyclic AMP. © 2000 Academic Press

Key Words: **axon growth; cyclic AMP; dorsal root ganglion; GAP-43; neurite; regeneration; tissue culture.**

INTRODUCTION

Elevated expression of the growth-associated protein GAP-43 is commonly observed in developing and regenerating neurons (9, 80, 87). GAP-43 can be present in the cell body and axon shaft, but is often concentrated in the growth cone (31, 54, 79). Association with membranes (81, 98), interaction with actin (33, 55), calmodulin binding (45), interaction with the G protein G_0 (86), and interaction with rabaptin-5 (62) suggest that GAP-43 may be involved in regulating growth cone motility and guidance. Indeed, some neurons depleted of GAP-43 display unusual growth cone motility and inappropriate responses to extracellular signals (1, 41, 76, 88).

Dorsal root ganglion (DRG) neurons provide a unique model to study axon regeneration because each neuron has both a peripheral and a central axon branch. After peripheral axotomy DRG neurons upregulate GAP-43 (72, 78, 92) and can regenerate their peripheral axon branches (26). After central axotomy DRG neurons do not upregulate GAP-43 (20, 73) and central regeneration within the spinal cord is poor.

The poor ability of central DRG axons to grow within the CNS environment may be due in part to inhibitory proteins present in CNS myelin (6, 18, 47, 51, 75). Many CNS neurons will regenerate only if they are provided with the alternative growth environment of a peripheral nerve graft (8, 22, 67). In the case of DRG central axon branches, robust regeneration through peripheral nerve grafts seems to additionally require that the neurons also suffer a lesion of their peripheral axon branches (21, 68, 69, see also 61). This indicates that both a favorable environment and an injury signal from the peripheral axon branch are necessary for regenerative growth of DRG axons. One consequence of peripheral (but not central) axotomy is that DRG neurons increase their expression of GAP-43 and transport it to both axon branches (72, 73).

The signaling mechanism associated with peripheral, but not central, axon injury which leads to changes in regenerative ability and increased GAP-43 expression is not well understood. The magnitude of GAP-43 induction is the same, no matter where along the peripheral axon branch injury occurs (43). This may be a general feature of peripheral, but not central, neurons (27). Prevention of peripheral target reinnervation by DRG neurons prolongs high GAP-43 expression (11, 19, 72). Exposure of DRG neurons to peripheral target cells *in vitro* causes a decrease in GAP-43 (5). Taken together, these findings suggest that contact with peripheral (but not central) target cells represses

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GAP-43 expression in DRG neurons and that peripheral axotomy relieves this repression.

This study investigates the regulation of GAP-43 expression and neurite outgrowth potential in dissociated adult rat DRG cultures. In such cultures, DRG neurons spontaneously extend neurites (36, 44, 82) and upregulate GAP-43 expression (74), consistent with the absence of putative repressor(s). We surveyed extracellular signals and signal transduction pathways to determine if any of these might mediate a target-derived GAP-43-repressive signal. We then correlated changes in GAP-43 expression with changes in the ability to carry out neurite outgrowth. Our findings demonstrate that at least one GAP-43-repressive pathway exists, but that activation of this pathway does not necessarily repress neurite growth.

EXPERIMENTAL PROCEDURES

Animals were adult female Wistar rats bred in our laboratories, in accordance with the standards of the Canadian Council on Animal Care. Reagents were obtained from Sigma–Aldrich Canada, unless otherwise noted. Procedures were carried out at room temperature unless otherwise noted.

Cell Culture

Adult rat DRG cells were dissociated and maintained *in vitro* using a modification of the method of Lindsay (44). DRG were obtained from 200- to 250-g rats euthanized by exposure to carbon dioxide. Approximately 40–48 DRG were dissected from each rat, rinsed in L15 nutrient medium, and cleaned of connective tissue. Tissue was dissociated by first incubating in L15 containing 0.1% collagenase (90 min, 37°C) and then adding trypsin to a final concentration of 0.02% (30 min, 37°C). Trypsin activity was quenched by the addition of horse serum (HS, produced locally) and dissociation was completed by trituration. The cells were washed in F14 medium (Life Technologies, Inc.) plus 10% HS and resuspended in 2 ml F14 plus 10% HS, and nigrosin-excluding neurons were counted using a hemocytometer. Standard density cultures for immunocytochemistry and cell ELISA were seeded at 500 neurons per well in 96-well plates. For cAMP assays, Western blots, and RNase protection assays, cultures were seeded at 5000 neurons per well in 24-well plates. For *in situ* hybridization, cultures were seeded at 12,500 neurons per 35-mm dish. All culture plates were precoated with a 0.1 mg/ml solution of poly-L-lysine.

In one series of experiments, cultures were seeded at a density of one neuron per well in 96-well plates to produce single neuron cultures. In these cultures, horse serum was omitted, and 5 μg/ml insulin, 5 μg/ml transferrin, 20 nM progesterone, 100 μM putrescine, and 30 nM selenium were added (14).

The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Half of the medium was aspirated and replaced with fresh medium every 2 days. Experimental reagents (described under Results) were included at the time of initial plating and in all subsequent feed medium, except as noted. Adult DRG neurons cultured by this technique are not separated from nonneuronal cells. Thus, in these cultures, the nonneuronal DRG cells (particularly Schwann cells) survive and proliferate.

Immunocytochemistry

Seven-day DRG cultures were rinsed in Hanks' balanced salt solution, fixed in cold methanol (−20°C, 30 min), rinsed again in phosphate-buffered saline, pH 7.4 (PBS), and then treated with a blocking solution consisting of 2% horse serum and 0.5% bovine serum albumin in PBS (60 min). This was replaced with either monoclonal antibody 9-1E12 against GAP-43 (72) diluted 1:5000 in blocking solution or monoclonal antibody AP20 against MAP2 (Sigma–Aldrich Canada) diluted 1:200 in blocking solution (45 min). After washing in PBS, antibody binding was developed using biotinylated anti-mouse IgG diluted 1:500 and then avidin–peroxidase diluted 1:500 (30 min each), followed by PBS washes. Peroxidase activity was localized by reacting in 0.05% H_2O_2 and 0.025% diaminobenzidine (10 min).

Western Blots

At 7 days, DRG cultures were rinsed in PBS and solubilized in 1% sodium dodecyl sulfate, and insoluble material was removed by centrifugation. Protein corresponding to 6000 neurons per lane was electrophoresed through a 12% acrylamide gel and then blotted to Immobilon P (Millipore Corp.) filter paper in a buffer containing 40 μM AMPSO and 20% methanol using a semidry blotting apparatus. The filters were blocked in a solution of 1% nonfat milk powder and 0.05% Tween 20 and then exposed to monoclonal antibody 9-1E12 (1:20,000) in blocking solution (30 min). The filters were washed in PBS and exposed to biotinylated-anti-mouse IgG diluted 1:5000 and then to avidin–peroxidase diluted 1:5,000 (30 min each), followed by PBS washes. Peroxidase activity was revealed by reacting in 0.05% H_2O_2 and 0.025% diaminobenzidine (10 min).

Cyclic AMP Assay

Cyclic AMP (cAMP) was assayed using cyclic AMP ^{3}H assay system (Amersham Canada) according to the manufacturer's instructions. Cultures were rinsed with PBS and then extracted twice with 0.5 ml ethanol (5 min each). Ethanol extracts were combined and then evaporated to dryness. The extract was dissolved in binding buffer and combined with cAMP-binding pro-

tein and [^{3}H]cAMP (2 h, 4°C). Free nucleotide was removed by charcoal adsorption, and the radioactive labeling of the binding protein complex was measured by liquid scintillation counting. The values obtained were used to calculate cAMP content in comparison to a standard curve constructed using known amounts of nonradioactive cAMP. Each assay was performed in triplicate.

Cell ELISA for GAP-43 Protein

The GAP-43 protein content of adult DRG cultures was measured by cell ELISA (74). Cultures were rinsed with Hanks' balanced salt solution and then fixed in cold methanol (−20° C, 30 min) and dried under vacuum (30 min). Excess protein-binding capacity in each well was blocked with 10% nonfat dry milk and 0.05% Tween 20 in PBS (1 h, 37°C). This was then replaced with 50 μl of blocking solution containing monoclonal antibody 9-1E12 against GAP-43 diluted 1:5000 (45 min). The wells were washed with PBS and filled with 50 μl of peroxidase-conjugated anti-mouse IgG diluted 1:500 in blocking solution (30 min) and then washed again with PBS. Peroxidase activity was quantified by adding 200 μl of a solution of H_2O_2 and *o*-phenylenediamine dihydrochloride (30 min) prepared from tablets according to the manufacturer's instructions. The reaction was stopped by the addition of 50 μl of 3 M hydrochloric acid. The absorbance of the solution at 490 nm was measured using a Spectramax 340 microplate reader. Each assay was carried out in sextuplicate.

On each 96-well plate, 24 of the wells were coated with partially purified GAP-43 to construct a standard curve. Partially purified GAP-43 (0–1.1 ng GAP-43 protein) was diluted to 50 μl in distilled water and added to wells (not precoated with poly-L-lysine) and allowed to bind overnight at 37°C. The wells containing GAP-43 standards were processed in parallel with the wells containing cultured cells and linear regression was used to construct a second order standard curve (74).

Nuclease Protection Assay for GAP-43 mRNA

Total RNA was extracted from DRG cultures using Trizol reagent (Life Technologies, Inc.), according to the manufacturer's instructions. Nuclease protection assays were carried out using a synthetic oligonucleotide probe (University Core DNA Services, University of Calgary, Calgary, Alberta, Canada) complementary to nucleotides 53–100 of GAP-43 mRNA (7). A recent database search revealed that this sequence had less than 60% homology to any other reported sequence. Control probes for cyclophilin and for 28S rRNA were also used. The oligonucleotides were end labeled using [γ-^{32}P]adenosine triphosphate (NEN Life Science Products, Inc.) using T4 kinase (New England Biolabs) and separated from free nucleotide using a Biospin chromatography column (Bio-Rad Laboratories). RNA extracted from samples was hybridized with probe overnight (30°C) and then digested (30 min) with a mixture of S1, RNase A, and RNase TI (Multi-NPA kit, Ambion, Inc.) according to the manufacturer's instructions. Digests were electrophoresed through gels containing 8 M urea and 12% polyacrylamide and then exposed to preflashed X-omat AR film (Kodak, Inc.) overnight.

Neurite Outgrowth Assay

For morphological studies of neurite outgrowth, dissociated DRG tissue was depleted of nonneuronal cells by low-speed centrifugation (50*g*) through a single-step gradient of 15% bovine serum albumin in L15 medium. The neuronal pellet was resuspended and cultures were established at 10% of normal neuronal density, under otherwise identical conditions, and examined at 12–48 h. To study neurite outgrowth in older cultures, 7-day primary cultures were passaged to secondary culture at 4% of normal density. Cultures for passaging were rinsed once with Puck's balanced salt solution then incubated in the same solution containing 0.001% trypsin (2 min). Trypsin was removed, growth medium was added, and DRG neurons were resuspended by gentle trituration, a process which removed existing neurites. Cells were replated into secondary culture in poly-L-lysine-coated 35-mm tissue culture dishes and allowed to settle and extend new neurites for 8–12 h. Primary or secondary cultures were fixed by adding glutaraldehyde solution to a final concentration of 0.5% (30 min). The cells were stained with 0.25% Coomassie blue.

Neurite outgrowth analysis was carried out using a Northern Eclipse imaging system (Empix Imaging, Inc.). The first 100 isolated neurons encountered on a defined track across the plate were used for quantification. Manual counts were made to determine the percentage of neurons which bore any neurites and the number of neurite branches per neuron. Based on methods employed by others (37, 50) the steps used to measure total neurite length per neuron included: (i) use of a threshold function to highlight neurites, (ii) manual electronic erasure of background and cell bodies, (iii) skeletonization of remaining neurite images to one-pixel diameter, and (iv) electronic measurement of total skeletal neurite length per neuron.

Uridine Incorporation

To assess the effects of the transcription inhibitors 5,6-dichlorobenzimidazol ribose (DRB) and actinomycin D on synthesis of RNA, 7-day DRG cultures were exposed to 0.1 μM [^{3}H]uridine (Amersham Canada) and 0–160 μM DRB or 0–100 μM actinomycin D for 1 h. The dishes were rinsed twice in Hanks' balanced salt solution (37°C), and RNA was extracted using

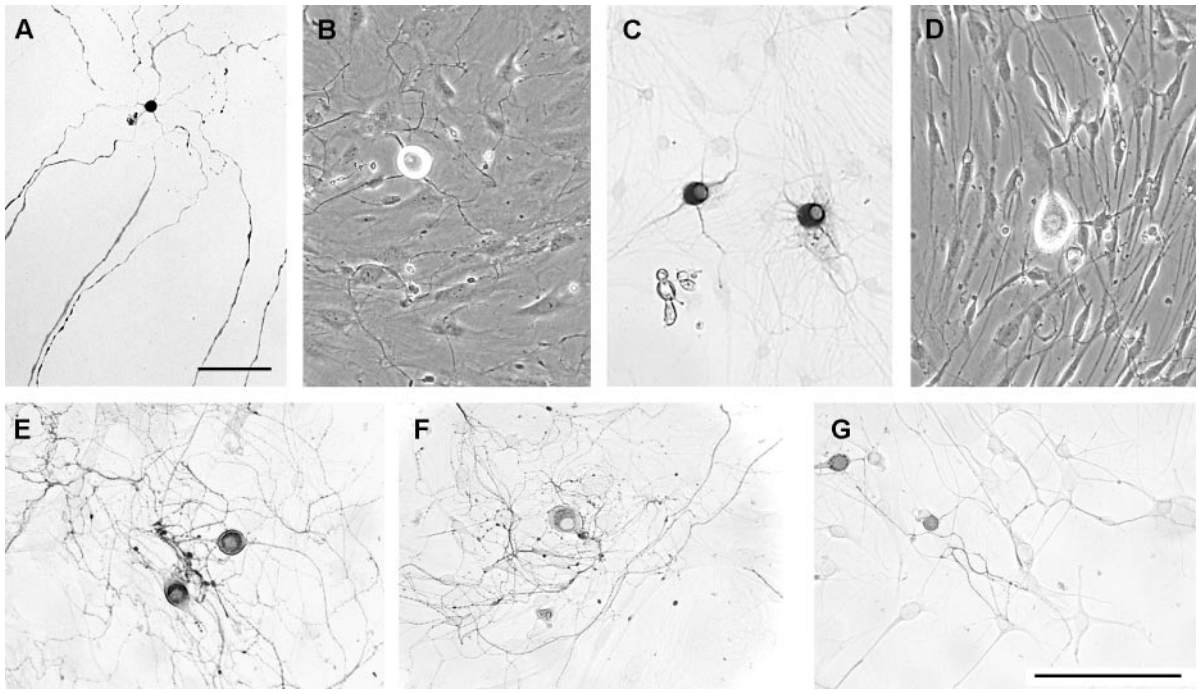

FIG. 1. Appearance of adult DRG cultures at 7 days. (A) GAP-43 immunocytochemistry of a DRG neuron in single-cell, serum-free culture. Bar, 100 μm. (B) Phase contrast image of standard density, control DRG culture. (C) MAP2 immunocytochemistry of control DRG culture. (D) Phase contrast image of DRG culture exposed to 0.5 mM dBcAMP. (E–G) GAP-43 immunocytochemistry of (E) control DRG culture, (F) DRG culture exposed to 0.5 mM dBcAMP, and (G) DRG culture exposed to 1.0 mM dBcAMP. Bar, 100 μm for (B–G).

Trizol reagent, as above. The RNA pellet from each sample was dissolved and dotted onto blotting paper, and the radioactivity in each sample was measured by liquid scintillation counting.

RESULTS

Appearance of Single Neuron Cultures

After 7 days in ultra-low-density, serum-free culture, we found that 40–60% of the wells in our 96-well plates contained living neurons. In most of these, only a single neuron was present, along with a small colony of Schwann cells or satellite cells. Fifteen wells were observed to contain only a single neuron, with no nonneuronal cells present. GAP-43 immunocytochemistry revealed that all neurons in ultra-low-density culture expressed high levels of GAP-43, including the 15 neurons which survived in complete cellular isolation (Fig. 1A), consistent with the concept that high GAP-43 expression is an intrinsic feature of DRG neurons.

Appearance of Standard Cultures

Seven days after seeding at standard density, adult DRG cultures contained large and small neurons situated upon a nearly confluent carpet of nonneuronal cells (Fig. 1B). Most of the nonneuronal cells had the morphological appearance of Schwann cells, but fibroblasts were also evident. Neurons could be unambiguously identified by virtue of their round or flattened MAP2-immunoreactive somata, with MAP2 immunoreactivity extending part way along proximal processes (Fig. 1C). GAP-43 immunocytochemistry revealed that virtually all neurons elaborated extensive neuritic arbors by 7 days. All detectable GAP-43 immunoreactivity was associated with neurons (Fig. 1E). Consistent with previous work (74), DRG neuron survival in such cultures was approximately 60% 2 days after plating, 50% 4r days after, and 40% 7 or 10 days after.

Chronic exposure to dibutyryl cyclic AMP (dBcAMP) caused a consistent change in the morphology of nonneuronal cells (Fig. 1D). However, the number and appearance of MAP2-positive neurons remained unchanged (not shown). The intensity of GAP-43 immunoreactivity declined with increasing doses of dBcAMP, although detectable GAP-43 immunoreactivity persisted even at 1 mM dBcAMP, the highest dose examined (Figs. 1F and 1G). Neurite outgrowth remained profuse, and GAP-43 immunoreactivity remained confined to neurons and their processes.

GAP-43 Protein Content of Cultures

Western blots of 7 day adult DRG cultures revealed that GAP-43 immunoreactivity was associated with a single band, previously characterized as containing authentic GAP-43 (72). Qualitative examination of Western blots indicated that the intensity of this band was

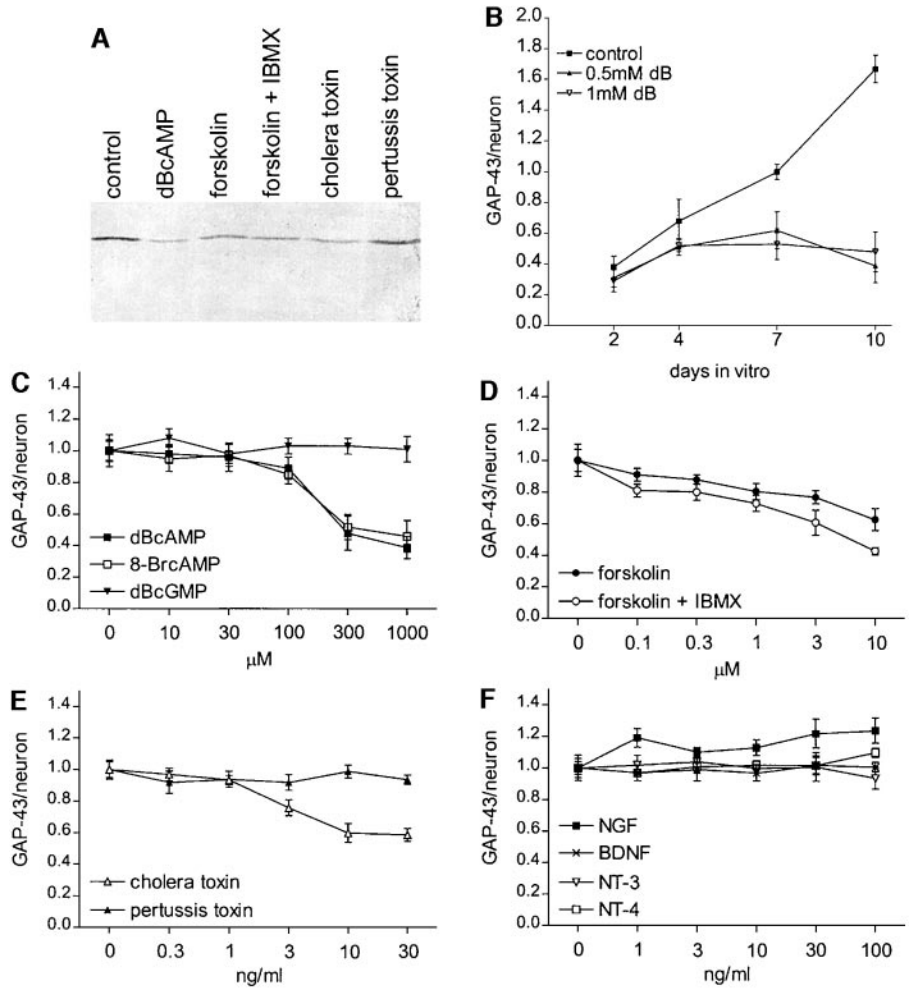

FIG. 2. Analysis of GAP-43 protein content of DRG cultures. (A) Western blot of protein extracted from cultures treated with 0.5 mM dBcAMP, 10 μM forskolin, 10 μM forskolin plus 50 μM IBMX, 10 ng/ml cholera toxin, or 10 ng/ml pertussis toxin for 7 days. (B–F) GAP-43 cell ELISA normalized to neuron number. (B) GAP-43 content of DRG cultures treated with 0.5 or 1.0 mM dBcAMP for 2 to 10 days. dBcAMP treatment at either dose caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) decrease in GAP-43 at 2, 4, 7, and 10 days. (C) GAP-43 content of DRG cultures treated with 0–1000 μM dBcGMP, dBcAMP, or 8BrcAMP for 7 days. The two cAMP analogs caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) decrease in GAP-43 at doses of 100 μM and above. (D) GAP-43 content of DRG cultures treated with 0–10 μM forskolin or 0–10 μM forskolin plus 50 μM IBMX for 7 days. Forskolin caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) decrease in GAP-43 at doses of 1 μM and above. Forskolin plus 50 μM IBMX caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) decrease in GAP-43 at doses of 0.1 μM and above. (E) GAP-43 content of DRG cultures treated with 0–100 ng/ml cholera toxin or 0–100 ng/ml pertussis toxin for 7 days. Cholera toxin caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) decrease in GAP-43 at doses of 3 ng/ml and above. (F) GAP-43 content of DRG cultures treated with 0–100 ng/ml NGF, BDNF, NT-3, or NT-4 for 7 days. NGF caused a significant ($P < 0.05$; ANOVA followed by Dunnett's test) increase in GAP-43 at doses of 1 ng/ml or above. Error bars indicate standard deviation.

decreased in cultures chronically exposed to dBcAMP. A similar decrease was seen in cultures exposed to forskolin, forskolin plus IBMX, or cholera toxin, but exposure to pertussis toxin had less of an effect (Fig. 2A).

Cell-ELISA was used to quantify the rise in GAP-43 content of DRG cultures over 10 days *in vitro*. As reported previously (74), a nearly threefold increase in GAP-43 content of control cultures was observed between days 2 and 10, even though neuronal survival decreases by half over this time. This rise in GAP-43 was effectively blocked by chronic inclusion of 1 mM dBcAMP or 1 mM 8-bromo cyclic AMP (8BrcAMP) (Fig. 2B). In dose–response experiments assayed at 7 days, the GAP-43 content of cultures was significantly lower in cultures chronically exposed to 0.1 mM either reagent, and inhibition of the GAP-43 increase reached 50–60% with 1 mM either reagent (Fig. 2C). GAP-43 content of cultures was not affected by dibutyryl cyclic GMP.

To determine whether elevation of endogenous cAMP would have the same effect as exogenous ana-

logs, we studied the effects of forskolin and cholera toxin. The cAMP content of 7-day control cultures was approximately 0.06 pg per microgram of total protein. Exposure to forskolin elevated cAMP levels 10- to 15-fold after 1 h. This effect gradually diminished when measured at longer exposure times, but remained significantly above controls even after 7 days (not shown). The effect of forskolin was potentiated by inclusion of 50 μM IBMX, a cyclic nucleotide phosphodiesterase inhibitor (not shown). Exposure of our cultures to 10 ng/ml cholera toxin raised cAMP levels 3- to 5-fold above control levels at 1 h (not shown). At longer time periods, the response of cAMP to cholera toxin was diminished and was often not statistically significant when assayed at 7 days. In the presence of 50 μM IBMX, the effect of cholera toxin on cAMP levels was more pronounced. Pertussis toxin or IBMX alone did not alter cAMP levels (not shown).

GAP-43 content of 7 day adult DRG cultures was lower by approximately 25% in cultures chronically exposed to 1 μM forskolin and by approximately 35% with 10 μM forskolin. The effect of forskolin was augmented by the addition of 50 μM IBMX, producing a reduction of GAP-43 content of nearly 60% at the highest forskolin concentration (Fig. 2D). DMSO vehicle, used for forskolin and IBMX, had no effect (not shown). The forskolin-induced reduction of GAP-43 content was also enhanced by a second phosphodiesterase inhibitor, Ro 20 1724 (Life Technologies, Inc.) (not shown). Forskolin, with or without phosphodiesterase inhibitors, did not affect neuronal survival. Chronic exposure of cultures to 10 ng/ml cholera toxin in culture medium resulted in a reduction of the GAP-43 content in 7-day DRG cultures of approximately 40% (Fig. 2E). Inclusion of pertussis toxin up to 30 ng/ml had no effect. Neither cholera toxin nor pertussis toxin affected neuronal survival.

To confirm that cholera toxin repression of GAP-43 was mediated by adenyl cyclase, we examined the effect of 200 μM SQ22,536 (RBI/Sigma), an adenyl cyclase inhibitor. This inhibitor caused an increase of GAP-43 protein when used alone and reversed the ability of cholera toxin to repress GAP-43 content of 7-day adult DRG cultures (Fig. 3A) without affecting neuronal survival. We also examined the effects of a competitive inhibitor of cAMP, dideoxyadenosine (DDA). Cultures chronically exposed to 100 μM DDA caused a small, but significant, increase in GAP-43 protein, but did not significantly reverse the repressive effects of cholera toxin (Fig. 3B). DDA had no effect on neuronal survival at this dose. These observations suggest that activation of G_s by cholera toxin represses GAP-43 levels by acting through adenyl cyclase and a downstream signaling step which is insensitive to DDA.

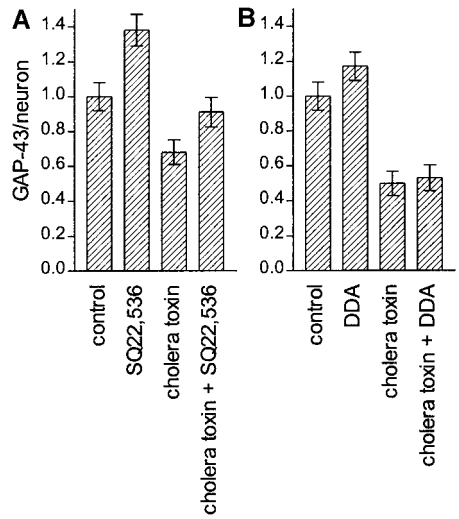

FIG. 3. Analysis of GAP-43 protein content of DRG cultures by cell ELISA normalized to neuron number. (A) GAP-43 content of DRG cultures treated with 200 μM SQ22,536, an adenyl cyclase inhibitor, for 7 days. SQ22,536 alone caused a significant ($P < 0.001$; ANOVA followed by Tukey's test) increase in GAP-43 and caused a significant ($P < 0.001$; ANOVA followed by Tukey's test) reversal of GAP-43 repression by 10 ng/ml cholera toxin. (B) GAP-43 content of DRG cultures treated with 100 μM DDA, a competitive inhibitor of protein kinase A, for 7 days. DDA alone caused a slight, but significant increase ($P < 0.01$; ANOVA followed by Tukey's test) in GAP-43 and caused a slight, but significant ($P < 0.05$; ANOVA followed by Tukey's test), reversal of GAP-43 repression by 10 ng/ml cholera toxin. Error bars indicate standard deviation.

Effects of Growth Factors and Other Reagents on GAP-43 Protein Levels

NGF and other neurotrophins appear to play a role in regulating some changes in gene expression which follow injury of DRG neurons (94, 96). Chronic exposure to NGF (Cedar Lane), BDNF, NT-3, and NT-4 (Alomone Labs) in a dose range from 1 to 100 ng/ml resulted in no detectable change in GAP-43 content of 7-day adult DRG cultures with the exception of a modest (approximately 20%) increase of GAP-43 in the presence of NGF (Fig. 2F). No significant change in survival in response to these neurotrophins was observed.

We attempted to activate other signaling pathways potentially related to the control of axon growth by inclusion of additional factors in the DRG cultures. Chronic exposure to the cytokines GDNF or CNTF (Alomone Labs) or LIF at several concentrations resulted in no detectable change in GAP-43 protein content of 7-day adult DRG cultures (not shown). IGF1 also had no effect (not shown). Precoating the culture surface with the axon-growth-promoting extracellular matrix protein laminin had no effect on GAP-43 expression (not shown). Chronic exposure to the phorbol ester β-phorbol myristate acetate (PMA), which would be expected to inactivate protein kinase C, did not

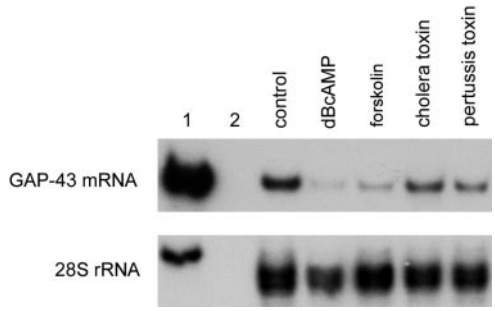

FIG. 4. Analysis of GAP-43 mRNA content of DRG cultures by nuclease protection assay. The first two lanes show the labeled GAP-43 or 28S RNA probes (1) without or (2) with nuclease treatment. Subsequent lanes show GAP-43 or 28S RNA probe protection by RNA extracted from control cultures and cultures exposed to 0.5 M dBcAMP, 10 μM forskolin, 10 ng/ml cholera toxin, or 10 ng/ml pertussis toxin for 3 days.

effect GAP-43 content of DRG cultures (not shown). Chronic exposure to 100 μM arachidonic acid also had no effect on GAP-43 (not shown). We included the nitric oxide-generating compound SNAP (Biomol Research Laboratories, Inc.) at a concentration of 1 μM, expecting to generate nanomolar concentrations of nitric oxide. This treatment also did not affect the GAP-43 content of our cultures (not shown).

Effect of cAMP Signaling on GAP-43 mRNA Levels

To investigate the mechanism of GAP-43 protein down-regulation, we measured GAP-43 message levels in nuclease protection assays. In samples from cultures chronically exposed for 7 days to dBcAMP, forskolin, or cholera toxin, GAP-43 mRNA levels were diminished in comparison to control (Fig. 4), although there was no corresponding decrease in 28S rRNA or cyclophilin mRNA (not shown).

Influence of cAMP Signaling on Neurite Outgrowth

Neurite outgrowth in our standard 7-day DRG cultures is too dense for measurement of individual arbors (Figs. 1B and 1E). To assess neurite outgrowth ability, DRG cultures were grown at 10% of the usual density and examined after 12, 24, or 48 h of primary culture. To examine growth potential after prolonged exposure to experimental reagents, DRG neurons were also examined 8–12 h after being replated into secondary culture at low density from a standard density 7-day primary culture. The efficiency of replating into secondary culture was approximately 90%, virtually all existing neurites were lost during passage, and the density of nonneuronal cells was greatly reduced.

The number of neurons initiating neurite outgrowth rose from under 20% at 12 h to over 80% at 48 h in low-density primary culture. Neurite outgrowth occurred from almost all neurons in low-density secondary culture within 8 h after replating, consistent with previous reports that most adult DRG neurons become primed for growth only after a delay following axotomy and removal to culture (82). The percentage of neurons initiating neurite growth was unaltered by exposure to dBcAMP during the initial 48 h of low-density primary culture. Neurite initiation was also unaltered if standard density primary cultures were exposed to dBcAMP for 7 days and dBcAMP remained in the medium during 12 h of subsequent low-density secondary culture (Fig. 5A). However, when the total length of the

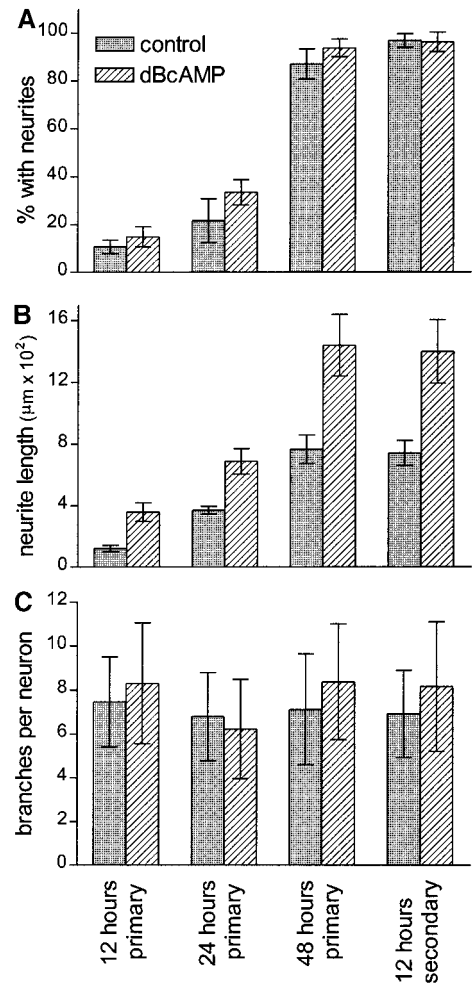

FIG. 5. Analysis of neurite growth in DRG cultures. (A) Number of neurons bearing neurites in primary DRG cultures after treatment with 0.5 mM dBcAMP for 12, 24, or 48 h or in secondary DRG cultures after treatment with 0.5 mM dBcAMP for 12 h. dBcAMP caused no significant change (Student's *t* test) in neurite initiation at any time point. (B) Total neurite length per neuron bearing neurites in primary DRG cultures after treatment with 0.5 mM dBcAMP for 12, 24, or 48 h or in secondary DRG cultures after treatment with 0.5 mM dBcAMP for 12 h. dBcAMP caused a significant ($P < 0.01$; Student's *t* test) change in neurite length at each time point. (C) Number of branch endings per neuron bearing neurites in primary DRG cultures after treatment with 0.5 mM dBcAMP for 12, 24, or 48 h or in secondary DRG cultures after treatment with 0.5 mM dBcAMP for 12 h. Neither time in culture nor dBcAMP caused a significant change (ANOVA) in neurite branch number.

neuritic arbor from each neurite-bearing neuron was measured, we found that length of neurite outgrowth was increased approximately twofold in primary and secondary cultures continuously exposed to dBcAMP (Fig. 5B).

Increased rate of neurite outgrowth, coupled with a decrease in neurite branching, has been used to define distinct modes of outgrowth in cultured DRG neurons (82). To examine this issue, we counted the number of branches that made up the neuritic arbors in primary and secondary cultures. The number of branches per neuron varied widely, but almost all neurons fell within the range of 3–15 branches per neuron. Number of branches was unrelated to total neurite length (not shown). We found that exposure to dBcAMP had no effect on branch number in primary or secondary cultures (Fig. 5C).

To determine if the effect of cAMP activation on neurite outgrowth was chronic or acute, we reversed exposure to dBcAMP, such that cultures exposed to dBcAMP for 7 days in standard density primary culture were replated for 12 h in secondary culture in control medium, and cultures grown for 7 days in control medium were replated for 12 h in medium containing dBcAMP. Acute exposure to dBcAMP in secondary culture caused an increase in neurite outgrowth which was independent of prior chronic exposure to dBcAMP (Fig. 6A). To determine if this rapid effect might involve rapid alteration of gene transcription, we examined secondary DRG cultures exposed to transcription inhibitors. The transcription inhibitor DRB was capable of reducing [^{3}H]uridine incorporation by greater than 95% (Fig. 6B), but was unable to block the growth stimulatory effect of either dBcAMP or cholera toxin (Fig. 6C). Similarly, actinomycin D also did not block dBcAMP enhancement of growth, although this reagent only reduced [^{3}H]uridine incorporation by approximately 75% (not shown).

DISCUSSION

In most adult DRG neurons expression of GAP-43 mRNA and protein are low or absent, but undergo a substantial increase 1 week following peripheral axotomy (34, 72, 92, 93) or removal to culture (36, 74). We show that adult DRG neurons express high levels of GAP-43 even when cultured for 7 days as single cells, and without the addition of serum or growth factors, suggesting that the rise in GAP-43 expression is not caused by extrinsic positive signals.

Axon growth following peripheral axotomy *in situ* is thought to occur only after a delay (12, 29, 52), and most cultured DRG neurons initiate long neurite growth only after a delay (82). These observations suggest that a change in protein synthesis, perhaps including GAP-43, is necessary for initiation of outgrowth in most DRG neurons.

We initially hypothesized that in intact adult DRG neurons high GAP-43 expression and the propensity for growth are repressed by target contact. Our studies were designed to search for influences which mimic the target-connected state, prevent the rise in GAP-43 which normally occurs when adult DRG neurons are removed to culture, and maintain DRG neurons in a metabolic state which does not support growth. We find that cAMP inhibits increased GAP-43 protein and mRNA expression. However, cAMP stimulates, rather than represses, DRG neurite outgrowth, even when GAP-43 levels are reduced.

Cellular Localization of GAP-43

Our standard density cultures included both DRG neurons and nonneuronal cells. Schwann cells can express GAP-43 in some circumstances (66, 71, 85, 89). Moreover, Schwann cell expression of GAP-43 can be decreased by cAMP (66, 71, 85). In our cultures, immunocytochemistry indicates that virtually all GAP-43 is confined to neurons. When GAP-43 cell ELISA is performed using purified Schwann cell cultures, the amount of immunoreactivity measured never exceeds approximately 10% of that found in our standard mixed neuron/Schwann cell cultures with similar Schwann cell density (P. L. Andersen and D. J. Schreyer, unpublished). Schwann cell GAP-43 is reportedly reduced by contact with neurons (71), so we expect Schwann cell GAP-43 to be even less significant in mixed cultures than in Schwann cell cultures. Together, these observations indicate that the reduction we see in GAP-43 can only be occurring in neurons.

Cyclic AMP Appears to Act Directly within Neurons

The observed reductions in GAP-43 could occur as a direct result of cAMP action within neurons or secondary to cAMP-induced changes within nonneuronal cells. In DRG cultures where greater than 90% of nonneuronal cells are removed by density gradient centrifugation and antimitotic treatment, we observe similar decreases in GAP-43 in response to cAMP (P. L. Andersen and D. J. Schreyer, unpublished). Further, we have observed similar reductions of GAP-43 in response to cAMP using neuronal cell line cultures devoid of nonneuronal cells (3). Taken together, these additional observations suggest that a direct effect of cAMP on the GAP-43 content of the neurons in our DRG cultures is likely.

Our studies of the effect of cAMP on neurite outgrowth used short-term, low-density, primary cultures of DRG neurons or DRG neurons acutely replated from standard density cultures. In both cases, virtually all nonneuronal cells were removed, and enhanced growth was seen in as little as 8 h. We can therefore say with certainty that the effects of cAMP on neurite growth were direct and not mediated by other cell types.

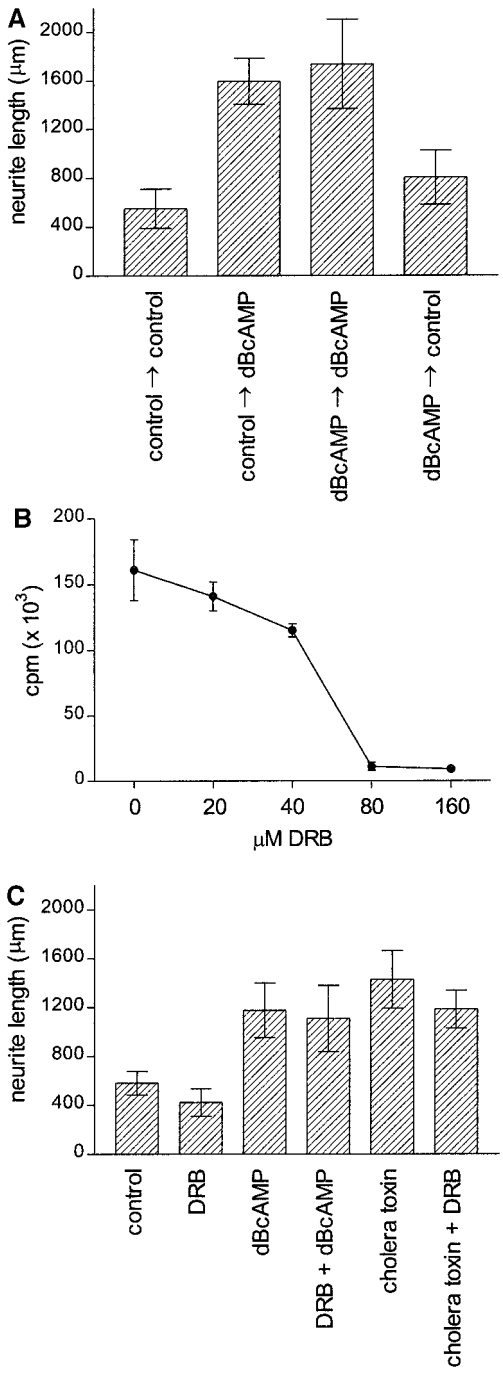

FIG. 6. Analysis of neurite growth in DRG cultures. (A) Total neurite length per neuron bearing neurites in secondary DRG cultures after treatment with 0.5 mM dBcAMP for 12 h. Secondary cultures were derived from standard density primary cultures which had or had not been treated with 0.5 mM dBcAMP for 7 days. dBcAMP caused a significant ($P < 0.01$; ANOVA followed by Dunnett's test) change in neurite length upon acute exposure to dBcAMP, regardless of prior chronic dBcAMP treatment. Prior chronic dBcAMP treatment caused no significant change in neurite length in secondary cultures not exposed to dBcAMP. (B) RNA synthesis assay in DRG cultures exposed to 0–160 μM DRB, a transcription inhibitor, for 1 h. Incorporation of uridine into RNA was significantly ($P < 0.01$; ANOVA followed by Dunnett's test) reduced by greater than 95% at DRB concentrations of 80 μM or greater. (C) Total neurite length per neuron bearing neurites in DRG cultures treated with 80 μM DRB for 12 h. DRB alone caused no significant change in neurite growth and caused no significant reversal of neurite growth stimulation by 10 ng/ml cholera toxin (ANOVA followed by Dunnett's test).

Linkage of Cyclic AMP to an Extracellular Signal

Chronic application of cAMP analogs or forskolin results in attenuation of the normal rise in GAP-43 protein in adult DRG cultures. Cholera toxin has the same effect, suggesting the presence of receptors linked to adenyl cyclase by G_s, a G protein target of cholera toxin. We have not yet exhaustively examined whether GAP-43 can be regulated by activation of G-protein-linked receptors for neurotransmitters, neuropeptides, opioids, prostaglandins, or hormones.

There can be convergence between signaling pathways utilizing cAMP and those utilizing neurotrophin receptors (13, 15, 28, 38, 39). Furthermore, the B (noncatalytic) subunit of cholera toxin can bind to GM1 gangliosides (59, 60), potentially influencing signaling through Trk receptors. In our experiments, exposure to BDNF, NT-3, or NT-4 did not down-regulate GAP-43 expression. NGF actually caused an up-regulation of GAP-43 protein, demonstrating that GAP-43 expression in control cultures, although high, is not yet at a maximum. Previous studies reported conflicting findings on whether NGF causes an increase or no change in GAP-43 mRNA in adult DRG cultures (36, 58). Regardless, we conclude that neurotrophins do not mimic the GAP-43-repressive activity of cAMP.

There can also be interaction between cytokine signaling and cAMP (10, 32, 64, 90). We found no ability of CNTF, GDNF, or LIF to reduce GAP-43 protein. We also investigated IGF1, which binds a receptor tyrosine kinase but also activates G_i (49) and may play a role in controlling GAP-43 in developing motor neurons (17), but found that it had no effect. We have not exhaustively surveyed all known growth factors to determine whether any others may enhance GAP-43 levels, as does NGF, or repress GAP-43 levels, as does cAMP.

Mechanism of Decrease in GAP-43 Protein

If the presence or absence of a target-derived repressor governs GAP-43 expression in DRG neurons *in situ*, it apparently influences protein levels by altering mRNA abundance (34, 92, 93). Changes in GAP-43 mRNA could reflect transcriptional regulation or could reflect altered mRNA stability (63, 65). Our studies using nuclease protection assays as a qualitative indicator indicate that cAMP also acts to repress GAP-43 mRNA. Cyclic AMP could therefore be the second messenger activated by a natural, target-derived repressor. But in this case as well, our techniques do not

allow us to distinguish whether the effect of cAMP is on transcription or on mRNA stability.

Mechanism of Increase in Process Outgrowth

We observed an increase in the total neurite length per neuron (of those neurons bearing neurites) following cAMP activation, but this was not due to any increase in neurite initiation or branching. Thus, cAMP appears to stimulate the rate of growth cone translocation.

The effect of cAMP on neurite growth was rapid, and rapidly reversible, and could occur in the presence of transcription inhibitors. This effect is therefore likely to be local and epigenetic. Regulation of growth cone motility has previously been reported to involve the action of cyclic nucleotides (46, 83). More recent studies indicate that cAMP can convert the response of growth cones to certain guidance factors from one of inhibition to one of attraction (57, 95, 84), perhaps acting through inhibition of members of the Rho family of small GTPases (48). Although our quantitative analysis of neurite outgrowth was done using a "neutral" polylysine growth substrate, there was serum present in the medium used in these studies. We are therefore unable to determine whether the observed effects of cAMP represented altered responses to extrinsic guidance molecules.

Necessity of GAP-43 for Process Outgrowth

We were surprised to find that repression of GAP-43 was correlated with stimulation of growth. We note that although cAMP blocks up-regulation of GAP-43, the protein never disappears altogether. This continuing low-level presence of GAP-43, also a feature of many uninjured adult DRG neurons (73, 93), may be sufficient to permit normal growth. Thus, even if cAMP appears to mimic some aspects of target repression of GAP-43, it apparently is insufficient to repress process outgrowth.

GAP-43 knockout mice display grossly normal nervous system development (88). Dendritic outgrowth from cultured hippocampal neurons occurs without GAP-43 (30, 31), and the neuron-like PC12 cell line can extend neurites in the absence of GAP-43 (4, 16). Furthermore, DRG neurons which remain devoid of GAP-43 following central axotomy can regenerate their central axon branches in the dorsal root, albeit at a slower rate (2). Thus, GAP-43 may be involved only in some types of process outgrowth. For example, GAP-43 appears to mediate the growth stimulatory effects of N-CAM acting through the FGF receptor (56), but is irrelevant for growth enhancement by laminin (88). Our present findings indicate that GAP-43 depletion does not inhibit outgrowth on polylysine.

Coincidence of Increased Neurite Growth with Decreased GAP-43

The effects of cAMP on GAP-43 and neurite growth appear to be dissociated. Stimulation of growth by cAMP occurs in the first 12 h of primary culture, before any injury-induced increase in GAP-43 can be detected (74, 92). Growth is also rapidly stimulated in secondary cultures of DRG neurons which were preconditioned to contain either high or low levels of GAP-43. Finally, cAMP acutely stimulates growth in the presence of transcription inhibitors. Thus, cAMP stimulation of growth appears to be insensitive to alteration of GAP-43 content in these neurons.

Functionally distinct adenyl cyclases can exist in different intracellular compartments (35). We have not examined whether regionalized cAMP signaling (i.e., growth cones vs cell bodies) can be correlated with either of the effects we observed.

Neurite Morphology

Increased neurite outgrowth in response to cAMP has been described for various neuron types (37, 42, 70, 78), but some authors have reported that long, regenerative growth *in vivo* is inhibited by cAMP (40, 53, 91). One possible reconciliation of these findings is that cAMP may induce neurons to switch their metabolism to support one mode of axon growth (branched, "sprouting"), at the expense of another (long, "regenerative").

In adult DRG neurons *in vivo,* neurotrophin-independent regenerative growth of injured axons can be differentiated from neurotrophin-dependent sprouting of uninjured axons (23, 24, 25). In culture, a subpopulation of adult DRG neurons initially exhibits slow, highly branched growth, but the entire population gains a capacity for faster, straight neurite growth after a delay (82). Acquisition of this ability to carry out fast, sparsely branched growth is enhanced by prior peripheral axotomy, but suppressed by transcription inhibitors (82). NGF potentiates the highly branched mode of growth (97).

Although we find that rate of outgrowth is increased by cAMP or by maintaining neurons in culture, neither manipulation has an effect on neurite initiation or branching. We are thus unable to demonstrate a change in mode of neurite outgrowth involving an altered pattern of branching as a consequence of either cAMP exposure or time in culture. It should be noted that our experiments were done using polylysine substrata, while previous studies describing distinct modes of growth examined neurite morphology on laminin substrata (82).

CONCLUSIONS

Our efforts to find a signaling pathway activated by a peripheral target factor and causing repression of

GAP-43 expression and growth potential in DRG neurons yielded unexpected results. cAMP does prevent a rise in GAP-43 protein in cultured, axotomized DRG neurons, consistent with the possibility that it mediates at least part of the action of the hypothesized repressor. However, cAMP causes rapid stimulation, rather than repression of neurite growth. Thus, a growth stimulatory mechanism is demonstrated which is independent of changes of gene transcription in general and of GAP-43 up-regulation in particular.

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