CELLULAR REGULATION OF NEURONAL BRANCHING

A DAY IN THE LIFE OF A GROWTH CONE

A Senior Thesis

By

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THESIS ABSTRACT

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What are the cellular mechanisms that govern when the outgrowing neurite of a neuron will branch? This research analyzed one possible biochemical pathway through which environmental and cellular signals are transduced into physical (motor) changes within the growth cone of an outgrowing neuronal process, and how this pathway might be linked to the probability of branching. The system used for these studies was the buccal nervous system of the pond snail, *Helisoma trivolvis*. Specifically, this research investigated the effects of stimulating the intracellular signaling cascades mediated by cyclic-AMP-dependent protein kinase A (PKA) by injecting individual *Helisoma* neurons, isolated into primary cell culture, with analogs of cyclic-AMP. Analogs used for these studies were either activators or inactivators of cyclic-AMP-dependent PKA. This research showed that activating cyclic-AMP-dependent PKA increased the likelihood of neuritic branching, and inactivating PKA had variable effects on the capacity of a neurite to branch depending upon the specific neuronal identity. Because this data suggests an optimal range of PKA activity within which a growth cone might branch, we present a model for the complex relationship between PKA activity, intracellular calcium, and changes in neuronal architecture.

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INTRODUCTION

The structure of the neuronal growth cone was first described over a century ago by Ramon y Cajal (1890). Neuronal growth cones are motile structures at the extending tips of outgrowing neurites that navigate the elongating process through the embryonic nervous system during development and the regenerating nervous system following injury (Davis et al., 1992). These structures ultimately develop into functional synaptic terminals at the sites of target contact. Growth cones serve as information-gatherers that communicate environmental signals to the neuronal process and, in turn, the cell body. They possess many finger-like extensions, called filopodia, that explore the cell's surroundings and detect these signals using membrane-bound receptors that carry out this sensory function. Signals detected by the filopodia are then transduced by the growth cone, eliciting changes in cellular behavior (Davenport and Kater, 1992). Therefore, the growth cone is involved in the coupling or integration of sensory and motor functions of the differentiating neuronal process (Kater and Rehder, 1995). During neuritic outgrowth there are occasions when the growth cone will bifurcate, or branch, sending out neurites in multiple directions. The general question addressed by the research within this thesis is concerned with the investigation of cellular and molecular mechanisms that determine when a neuronal process will branch. For the purposes of my project I have used identified neurons isolated from the buccal ganglia of the aquatic snail *Helisoma trivolvis* and plated into cell culture.

Previous studies on the behavior of nerve growth cones have given insight into some of the signals that might govern alterations in growth cone dynamics as neuronal processes traverse the nervous system and contact specific targets. Environmental or sensory cues such as chemical signals in the neuron's environment, either *in vitro* or *in vivo*, have been shown to affect neuronal growth and branching. For example, neurotrophic factors and growth factors, most notably nerve growth factor (NGF), enhance neuronal outgrowth *in vivo* and can guide neurite extension in cell culture (Menesini-Chen et al., 1978; Gundersen and Barrett, 1980). In addition, cell adhesion molecules such as

fasciclin II (fas II) of *Drosophila* and *Aplysia* cell adhesion molecule (apCAM) can affect branching in neuronal processes through the activation of signal transduction pathways within the cell. It has also been well established that intracellular calcium levels and the extracellular signals that modulate them, like the neurotransmitter serotonin (5-HT), play important roles in axonal elongation and growth cone motility in *Helisoma* neurons (Kater and Mills, 1991). Motile growth cones have higher free calcium levels than those growth cones whose motility has ceased or has been transformed into a stable state (Cohan, Connor, and Kater, 1987). The addition of serotonin to the neuronal culture medium inhibits neuritic extension in the some *Helisoma* buccal neurons, such as B19, by causing increases in intracellular calcium levels. This effect was not present in other buccal neurons, for example B5 (Haydon, McCobb, and Kater, 1987). Studies have also been conducted indicating that cAMP-elevating agents applied to the neuron suppress neurite elongation and growth cone motility, but upon exposure to calcium channel blockers, the inhibitory effects of these neuromodulators were abolished (Mattson et al., 1988). These studies also found that some neurons require calcium influx in order for cAMP to suppress neuritic growth while others do not.

It is clear that alterations in a growth cone's cytoskeletal structure (primarily actin) underlie changes in that growth cone's dynamics, motility, and neurite elongation (Smith, 1988). However, little is understood about the biochemical pathways that couple sensory cues to these mechanical properties underlying branching, the mechanism of sensory-motor coupling. Changes in several intracellular biochemical pathways, such as the cascade of events affecting the activity of cyclic-AMP-dependent protein kinase A (PKA), have been implicated in the regulation of growth cone differentiation and structural changes (Forscher et al., 1987). Studies of several animal systems have implicated the second messenger cyclic-AMP in the regulation of the growth cone sensory signal transduction leading to the cytoskeletal alterations that cause branching. First, studies of learning and memory in *Aplysia* demonstrated that increased cyclic-AMP levels in neurites caused a reduction in a

cell adhesion molecule, apCAM, which in turn elicited long-term structural changes, including neuronal branching (Zhu et al., 1995). Also, the laboratory of Corey Goodman at the University of California/Berkeley has studied "dunce" (*dnc*) mutants of the fruit fly *Drosophila melanogaster* whose neurons possess no active phosphodiesterase. Phosphodiesterase is the enzyme that breaks down cyclic AMP, and without it, cyclic-AMP builds up inside the cells and leads to increased branching (Budnik et al., 1990). This elevated level of cAMP in *dnc* flies also caused a decrease in the expression of the cell adhesion molecule fasciclin II which may couple changes in cAMP to alterations in branching and synapse formation at the *Drosophila* neuromuscular junction (Schuster et al., 1996).

The focus of this thesis is on the cellular mechanisms that mediate branching in a growing neuron, specifically whether alterations in the activities of cyclic-AMP-dependent protein kinase A affect the probability of branching. The neural system used for research in the laboratory of Mark Zoran at Texas A&M University is the buccal nervous system of the pond snail, *Helisoma trivolvis*. Neurons of this system are used extensively to study neuron-target interactions, addressing the mechanisms governing synapse formation between motoneurons and their specific muscle targets. In the Zoran lab, we are primarily interested in synaptogenesis, the process involving the transformation of growth cone form and function as this motile tip becomes a functional nerve terminal. In *Helisoma,* some motoneurons, such as buccal neuron 19 (B19), are highly selective in synapse formation and require contact with appropriate targets prior to synaptic competence (Zoran et al., 1990). It has also been recently discovered that, following interaction with appropriate muscle targets in cell culture, *Helisoma* growth cones have an increased likelihood of branching (Zoran et al., 1996). As mentioned above, the intracellular calcium levels in *Helisoma* neurons have previously been implicated in the regulation of growth cone behavior (Cohan, Connor, and Kater, 1987). In other studies, interaction of *Helisoma* neurons with specific targets was found to elicit a change in the neuronal influx of calcium,

altering both resting levels and action potential-evoked influxes. These alterations in calcium homeostasis depend upon the activation of cyclic-AMP-dependent protein kinase A (Funte and Haydon, 1993).

The current project tests the hypothesis that alterations in neuronal cyclic-AMP levels in these motoneurons are an important regulatory mechanism involved in the direct modulation of neuronal arborization (branching patterns of identified neurons). I have studied the effects of several cyclic-AMP analogs (specific activators and inactivators of cyclic-AMP-dependent PKA) on neuronal branching. Injecting neurons with these analogs either increases or decreases the levels of cyclic-AMP-dependent PKA activity and the resulting effects on neuritic branching were investigated using image analysis.

MATERIALS AND METHODS

I. Introduction to the *Helisoma* System

The system used for this neurophysiological research was the albino pond snail, *Helisoma trivolvis*. These molluscan neurons are very large relative to body size and can readily be removed from the nervous system for study in cell culture. Also, snails can be maintained in large numbers in laboratory aquaria and they reproduce quite rapidly, making a constant supply of animals readily available. Adult, laboratory-reared snails were maintained in freshwater aquaria at room temperature and were fed a daily diet of trout chow and fresh lettuce. Buccal neurons B19 and B5 innervate the muscles in the snail's buccal mass and esophagus, respectively, and mediate feeding behavior. These neurons were isolated from buccal ganglia and used for all studies.

II. Spherical Neurons in Culture

Buccal ganglia were dissected from the animal, then B19 and B5 neurons were isolated and plated into cell culture as previously described in Hadley et al. (1985) and Haydon and Zoran (1991). In short, snails were deshelled and anesthetized in Listerine for four minutes, then rinsed in sterile antibiotic saline solution (ABS) prior to dissection. Snails were dissected in a silicone-coated glass dish filled with ABS and visualized with the aid of a dissecting microscope. The buccal ganglia, once dissected, were placed into a sterile solution of defined medium (DM). Defined medium is a solution of 50% Leibowitz-15 (Gibco) to which 0.15 mg/mL L-glutamine and 4X salts were added [40 mM NaCl, 1.7 mM KCl, 1.5 mM $MgCl_2$, 4.1 mM $CaCl_2$, 10 mM Hepes (pH 7.3, 130 mOsm)]. Ganglia were then incubated in a solution of 0.2% trypsin (2 mg/mL trypsin - type III; Sigma Chemical, St. Louis, MO - in 2 mL DM) for 18 minutes to weaken the ganglionic sheath for ease in extracting neurons. The ganglia were then transferred to a silicone-coated

dissecting dish (stained with India ink to promote ease in viewing ganglia) containing a hyperosmotic solution of defined medium (4.5 mL DM + 0.5 mL 4X salts solution). Ganglia were pinned out in the dish and individual neurons were identified, then a small incision was made in the ganglionic sheath encasing the neurons. Single neurons were physically isolated using a micro-syringe-controlled glass micropipette, previously coated with sterile snail hemolymph to deter the adhesion of individual neurons to the walls of the pipette during extraction. In the general experimental paradigm (Fig. 1), isolated neurons were left to incubate at 25°C (in high humidity) for two days on BSA-coated plastic (Falcon 3001) culture dishes in 2 mL of conditioned medium, CM. Conditioned medium is DM that has been previously conditioned by three days of incubation with *Helisoma* central ring ganglia (2 ganglia per mL) which produce brain-derived growth-promoting factors. On day two of neuronal incubation, neurons in BSA-coated dishes were transferred to poly-L-lysine (PLL) coated plastic (Falcon 1008) culture dishes using a similar micropipette procedure to the one used for cell isolation. Cells were left to adhere to the dishes for 1-2 hours prior to injection with cAMP analogs or control solutions.

Up to this point, the preparation for each type of experiment was essentially identical. The protocol differs henceforth and will be described in sections depending upon the experiment. Three kinds of experiments were carried out in this project: chronic (long-term) studies of the effects of injecting cyclic-AMP analogs in to freshly-plated neurons, studies investigating the effects of acute injection of analogs after neurons had begun to extend neurites, and studies of the effects of acute injections on neurons prior to growth cone contact with muscle targets in culture. Plastic dishes were used for chronic and acute injection studies, but the neuron-muscle co-culture studies were conducted using glass-bottomed (Nunc) culture dishes in order to promote optical resolution during image analysis of muscle-contacted neurons following fluorescence staining.

Fig. 1:

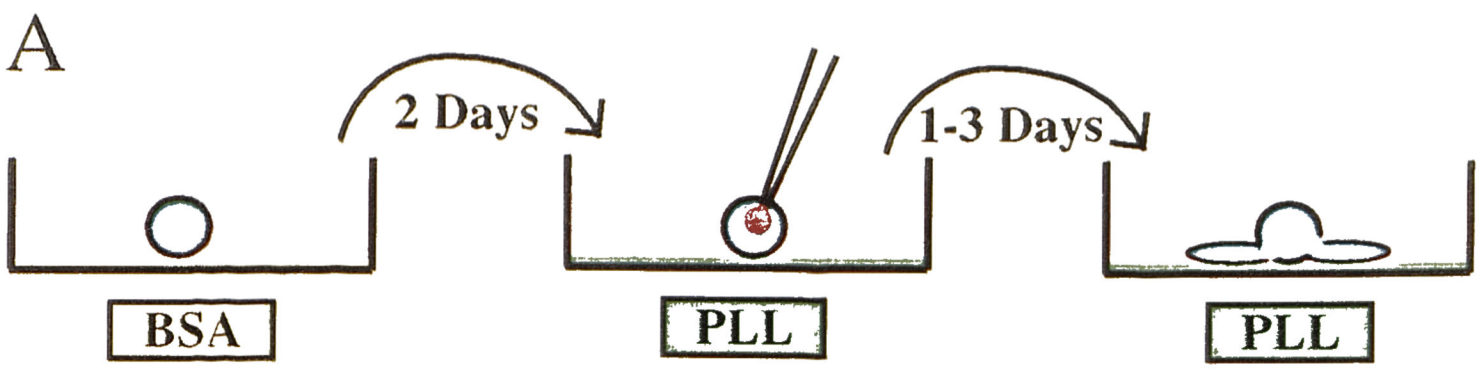
(A) Paradigm for Cell Culture Experiments. Cells were isolated and cultured for three days in plastic culture dishes that had been previously coated with BSA. After three days, cells were transferred into PLL-coated plastic or glass dishes and left to adhere. Cells were then injected with the appropriate cAMP analog or a control solution of 1% DMSO.

(B) Image analysis apparatus. Image analysis was conducted using a video-linked microscope and Macintosh Framegrabber Software.

(C) Neuronal outgrowth. This phase-contrast micrograph illustrates a spherical B19 neuron in culture one hour after plating onto an adhesive substrate.

(D) The same neuron as in panel C with extending growth cones three hours post-plating.

(E) Again, the same neuron with advancing growth cones and new neuritic arbors five hours post-plating. In all panels the cell body of the neuron is approximately 50 μm in diameter.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1-3 Days
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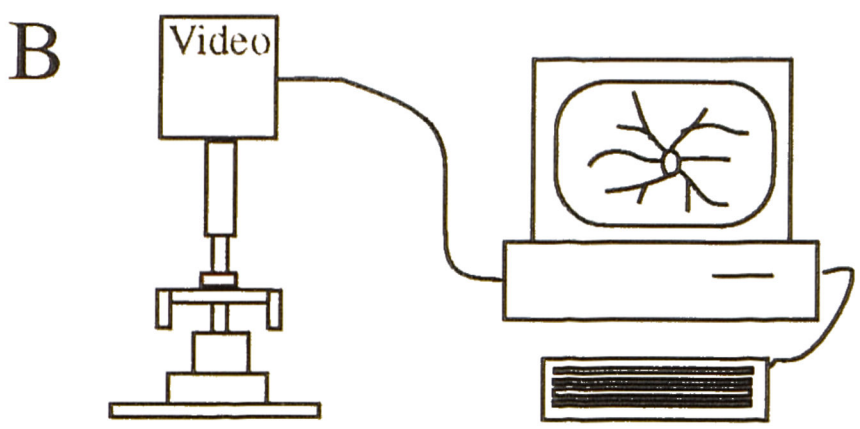
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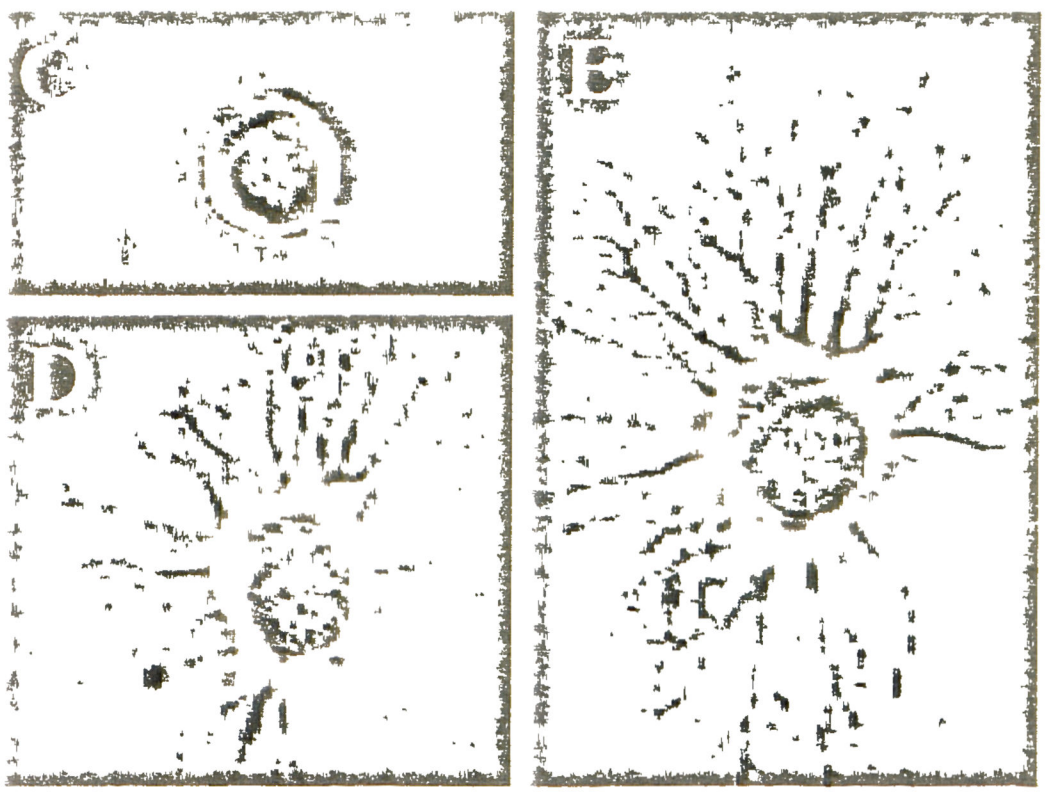
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III. Chronic Studies using cAMP Analogs

After neurons had sufficiently adhered to the PLL-coated plastic dishes, individual neurons were injected with a solution of 100μM Sp-cAMP (adenosine-3',5'-cyclic monophosphate, Sp isomer) using a Picospritzer that puffs solution in to the cell to increase the cell volume by approximately 10%. Injection studies were also conducted using Rp-cAMP (adenosine-3',5'-cyclic monophosphate, Rp isomer), 8Br-cAMP (adenosine-3',5'-cyclic monophosphate, 8-bromo sodium salt), and control solutions. Controls for this experiment were injected with 1.0% dimethyl sulfoxide solution (10 mL DMSO (Sigma)/1 mL sterile dH_20), the carrier solution for the cAMP analogs. Solid Sp-cAMP, Rp-cAMP, and 8Br-cAMP (Calbiochem) were dissolved in 1% DMSO to yield 100μM working solutions used for injection. Neuronal outgrowth usually began within the first 24 hours after plating and was imaged via time-course analysis over a period of three days, described below. Images were analyzed statistically following 48 hours of outgrowth for the percentage of cells initiating growth, the number of branches per millimeter of neurite, and the number of varicosities (synapses) present along the neurite.

IV. Acute Studies of the Effects of PKA Activity on Growth Cones

Individual neurons were isolated and cultured as described in part II. Cells were then transferred to glass-bottomed PLL-coated dishes, left to adhere for 1-2 hours, and observed for the onset of neuronal outgrowth. Once growth cones had emerged, cells were injected with 100 μm Sp-cAMP or Rp-cAMP as described in part III. Cells were observed and images captured before, at, and after injection at 1-2 hour intervals for a period of up to 24 hours in order to observe any changes in outgrowth rate or branching post-injection.

V. Neuron-Muscle Co-Cultures

Muscle fibers were dissociated from the supra-lateral radular tensor muscle (SLrT) of the buccal musculature of *Helisoma.* The SLrT muscle is an appropriate target of neuron B19 and is therefore used in conjunction with B19 in co-culture to study the effects of neuron-muscle interactions on branching. The buccal mass was dissected from the animal just after ganglia were dissected, and the individual SLrT muscle groups were removed from the mass. Muscles were treated with three successive washes in antibiotic saline, then placed into a sterile glass 5-mL test tube that had been pre-coated with BSA. Muscles were then dissociated into individual fibers through a 12-hour incubation in 2 mL of DM containing 4 mg collagenase/dispase (Boehringer-Mannheim) at room temperature. Muscles were then transferred into a 32°C water bath and left to incubate for 5-8 hours. After incubation, dissociated muscle fibers were diluted with DM, centrifuged to pellet the fibers, and washed two times with DM through centrifugation at 1000x g for 5 minutes. The muscle fibers were collected and transferred to a BSA-coated plastic culture dish (Falcon 1008), from which they were subsequently transferred into PLL-coated glass-bottomed dishes. Prior to plating neurons onto the glass-bottomed dishes, individual muscle fibers were transferred to ensure adhesion to the substrate prior to neuronal transfer. Spherical neurons were then transferred into the dish adjacent to individual muscle fibers. Neuron-muscle co-cultures were incubated at room temperature until neuronal outgrowth had been initiated. Just prior to the establishment of neuron-muscle contact, neurons were injected with cAMP analogs or DMSO control solution and image analysis was performed at 72 hours post-injection.

VI. Image Analysis

Cell cultures on plastic or glass-bottomed dishes were visualized through phase-contrast microscopy or epifluorescence microscopy using an Olympus inverted microscope. Images were captured using a JVC-ccd video camera attachment and/or Olympus 35mm camera attachments. Image capture was performed using a Macintosh computer with RasterOps videoboard running Framegrabber software. Video images/files were saved on the computer hard drive and were later archived to an APS removable 230 megabyte hard drive. Images were then analyzed using Adobe Photoshop software. Morphological features of the neurites were analyzed manually using digitized images or 35mm film prints.

VII. Statistical Data Analysis

For each cell, 2-4 sample neuritic regions of interest (ROI's) were analyzed. The number of original neurites, neuritic length in millimeters, number of neuritic arborizations per millimeter, and varicosities per millimeter were calculated for each ROI. Results from these property measurements of the individual ROI's were averaged and were tabulated for each type of cell under each experimental condition. Mean values, standard deviations (S.D.), and standard error of the mean (S.E.M.) were computed for each group. All data were presented as mean values plus or minus the standard error of the mean. Data were compiled in Microsoft Excel Version 5.0 spreadsheets and statistical analyses were performed using this program or Statview 4.1 software (Abacus). Two-tailed student t-tests were used to compare the paired experimental and control group means. Comparisons within and between groups over time were made using ANOVA followed with Fisher's paired least significant difference (PLSD) test. Statistical significance was represented as $p<0.05$.

RESULTS

General Paradigm

To formulate a general paradigm for this project, a series of descriptive studies of nerve growth cone development in cell culture were performed. In these morphological studies it was determined whether neurons initiate outgrowth faster if isolated into non-adhesive culture conditions one, two, or three days prior to plating into neurite outgrowth permissive conditions (Fig. 2). From these studies, I concluded that two days of culture provided sufficient percentages of cells with neurite outgrowth (65% of cells plated). This outgrowth of identified neurons usually began within 24 hours of plating onto an adhesive substrate (PLL-coated dishes) in the presence of conditioning factors. The rates of elongation of B19 neurites were analyzed over the first hours of growth cone emergence from the cell body (Fig. 3). These neuronal growth cones of B19 extended at fairly constant rates (approximately 3 μm/hour) during the initial 8 hours of neurite outgrowth. These rates of elongation increase dramatically at later stages (>6 hours post-plating) of cell culture. Therefore, this experimental protocol provided a population of neurons that could be approximately synchronized in their initiation of outgrowth.

We have hypothesized that activating cyclic-AMP-dependent protein kinase A (PKA) through injections of cells with cyclic-AMP analogs will lead to an increase in neuritic arborization. Also, we have hypothesized that inactivating PKA through injections with inactivating analogs will effect a decrease in neuritic branching. Thus, we conducted tests of these hypotheses using the paradigm of synchronized *Helisoma* neurons extending neuritic processes in culture.

Results of Chronic Studies

The studies involving 48 hour (chronic) outgrowth following neuronal injection were designed to investigate the effects of increased or decreased levels of cyclic-AMP-dependent protein kinase A (PKA) activity on neuritic outgrowth. These observations were conducted on cells injected before outgrowth had begun. Control data from these experiments (i.e., cells injected with 1% DMSO) support the findings of previous studies in which B19 neurons alone in culture (sans injection) possessed a baseline frequency of branching of approximately 10-15 branch points per millimeter (Zoran et al., 1996). This illustrated that cells injected with DMSO exhibit similar behavior to non-injected cells and that injection alone does not elicit deleterious effects on the propensity of a neuron to branch. Since injection of DMSO caused no particular ill effects, we went on to ask what effects injection of various analogs of cyclic-AMP had on cell outgrowth. That is to say, how does specifically activating or inactivating cyclic-AMP-dependent PKA affect the baseline rate of neuronal branching?

Effects of Sp-cAMP and 8Br-cAMP on neuron B19

Injecting 100 μM 8-bromo-cAMP into isolated B19 neurons caused an increased frequency of neuritic branching. The mean number of branch points per millimeter of neurite (± S.E.M.) effectively doubled from 13.9 (± 0.9; n=15) to 26.3 (± 2.1; n=8). These mean branching values for control (DMSO-injected) and 8Br-cAMP-injected neurons were significantly different ($p < 0.0001$). In support of this observation were results using a similar cAMP analog, Sp-cAMP. This PKA activator, also caused a significant elevation in branching above the control baseline rate (Fig. 4). The average number of branch points per millimeter in the Sp-cAMP-injected neurons almost doubled to a value of 23.0 (± 4.0; n=10). These results demonstrate that injection of cyclic-AMP analogs, known to elevate

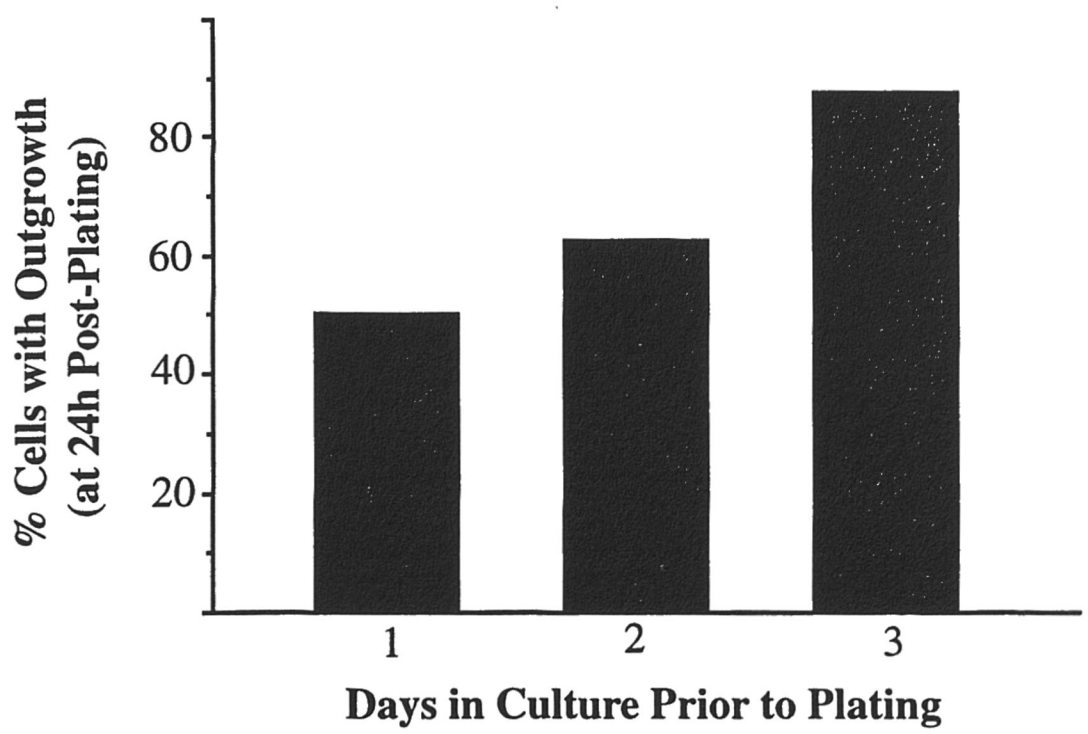


Fig. 2. Percentage of cells with neuritic growth at selected durations of cell culture. Although the percentage of cells with neurite outgrowth increased with time in culture prior to plating, the best protocol (based on experimental duration and synchrony of outgrowth) was determined to be 2 days of initial outgrowth-restrictive culture.

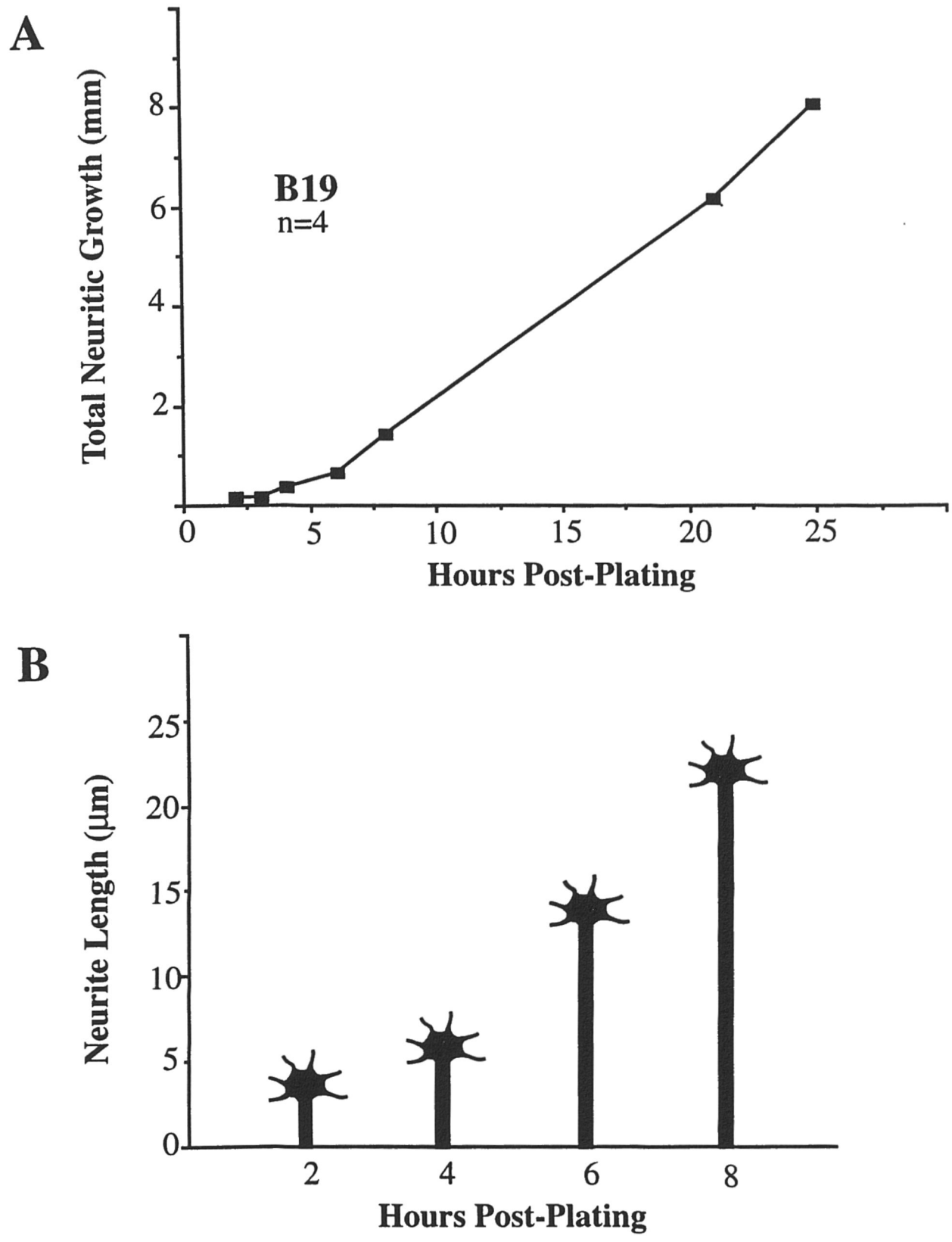


Fig. 3. Basal Neuritic outgrowth rates in cell culture (A) Total neuritic outgrowth of motoneurons in cell culture. Motoneuron B19, 2 days in culture, was viewed with phase-contrast microscopy and the total neuritic length was measured at selected time points. The earliest growth was seen at 2 hours post-plating onto an adhesive substrate. Extensive outgrowth was observed 20 hours post-plating.**(B) Average rate of neurite extension represented as neuritic length versus hours post-plating.** On average, neuron B19 extended neurites at approximately 3 µm per hour. At this rate, most growth cones have navigated some 25 µm from their points of origin at the cell body in the first 8 hours following plating onto an adhesive substrate.

PKA activity, caused an approximate two-fold increase in the probability of branching in neuron B19 (Fig. 5).

The effects of these PKA-activating cAMP analogs on the presence of varicosities (i.e., enlarged neuritic regions containing synaptic vesicles and thought to be sites of neurotransmission) were less consistent than the previous effects on branching. However, statistical comparison of varicosities in the baseline control groups and the Sp-cAMP-injected cells revealed significant differences ($p < 0.005$). No effect was observed on the number of original neurites extended from the cell body following these injections (see Table 1).

Effects of 8Br-cAMP and Sp-cAMP on neuron B5

Previous studies have shown that neuron B5 exhibits opposite effects from those of B19 in several cellular processes thought to be mediated through cAMP-dependent mechanisms. For instance, serotonin has been shown to suppress neurite elongation in B19 but not in B5 (Haydon, McCobb, and Kater, 1987). Also, B19 and B5 differ in their strategies of synapse formation (Zoran et al., 1996) and in their electrophysiological properties (Price and Goldberg, 1993; Achee and Zoran, 1996). Based on these reports, it was surprising to find in this study that the PKA activators had similar effects on B5 and B19. Injecting B5 neurons with 8Br-cAMP elicited a significant increase in branching above baseline B5 values ($p<0.02$; Fig. 5, Table 1), and injection of Sp-cAMP caused similar results. Again, two PKA-activators elicited similar effects on branching behavior in neuron B5. The PKA activators used in these studies had no apparent effects on the number of varicosities per millimeter in B5 compared to control values. It should be noted that the initial frequencies (set-points) of branching, varicosities, and original neurites produced were each significantly greater in B5 controls than B19 controls (see Table 1). The mechanisms responsible for these intrinsic differences in neuronal architecture between specific neurons remain unclear.

Effects of Rp-cAMP on B19 and B5

To further address the effects of PKA on branching patterns in chronic experiments, neurons were injected with Rp-cAMP, a cyclic-AMP analog and specific inactivator of cyclic-AMP-dependent PKA. After obtaining results from the injections of PKA activators into neurons, we were interested in finding out if antagonistic effects to those described above would be elicited by injecting an isomer of cAMP that specifically inactivates PKA. On the contrary, Rp-cAMP injection had no notable effects on branching in B19 with the mean number of branch points per millimeter being 16.4 (± 3.5; n=8) in Rp-cAMP-injected cells and 13.9 (± 0.9; n=15) in controls. (Fig. 5). Interestingly, the number of varicosities per millimeter increased significantly in B19 neurons ($p<0.005$; Table 1). In addition, Rp-cAMP caused a marked elevation in the likelihood of B5 neurons to both branch and produce neuritic varicosities (Fig. 5).

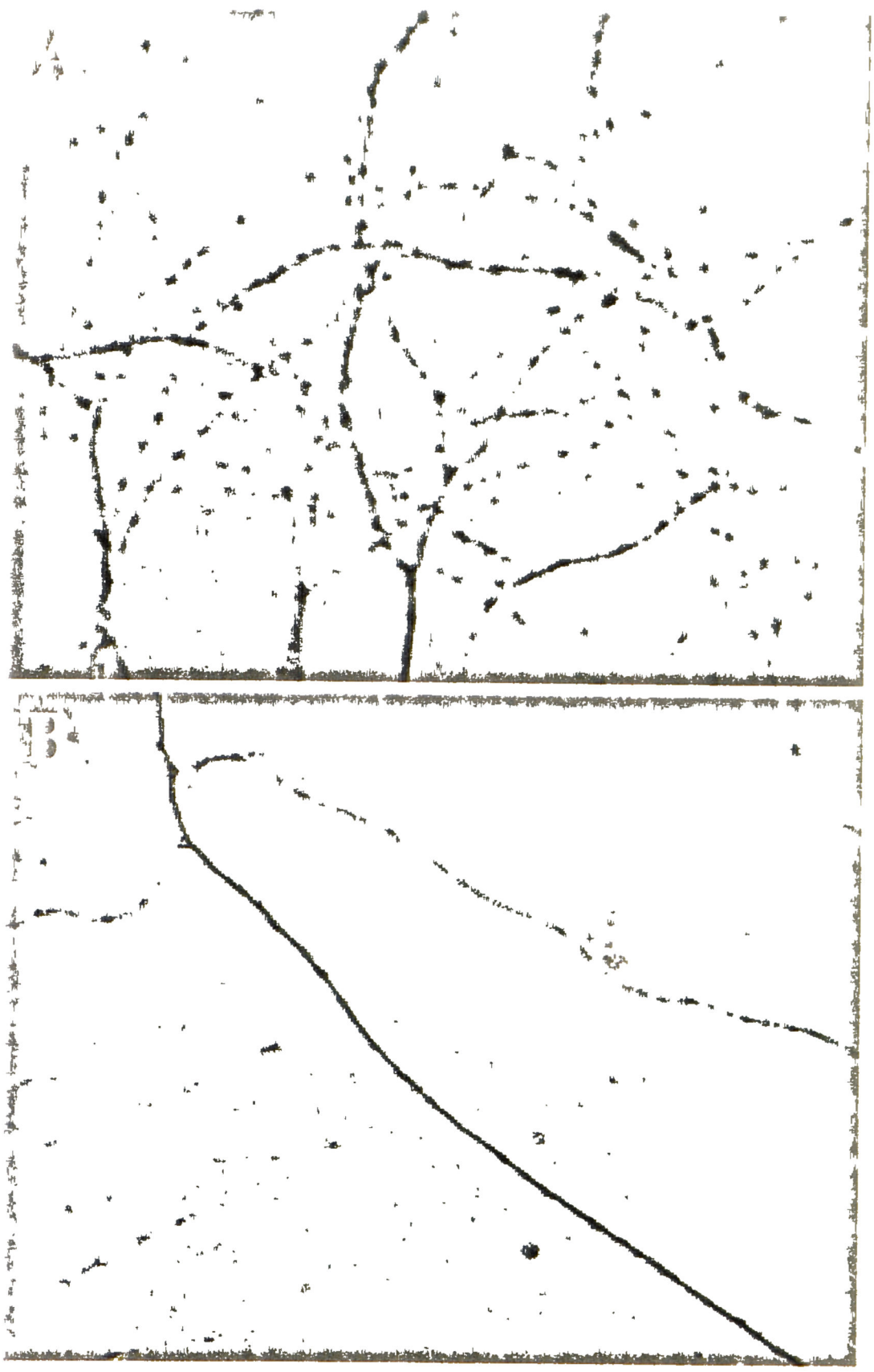
Results of Acute Studies

Acute experiments increasing or decreasing the activity of cAMP-dependent PKA on neuritic arborization were performed by injecting cAMP analogs directly into the cells that had previously initiated neurite outgrowth. Rates of neuritic outgrowth were not noticeably increased or decreased with respect to a linear growth rate in controls (Fig. 6). The average rate of neurite elongation for control neurons B19 was 185.0 (± 44.1; n=10) mm/hour; whereas, elongation rates for Sp-cAMP-injected cells and Rp-cAMP-injected cells were 214.3 (± 41.6; n=7) and 166.7 (± 44.1; n=3) mm/hour, respectively.
It is worth noting that these extension rates were calculated from growth cones that exhibited little or no branching after injection with analogs in order to assure linearity of

Fig. 4. Comparison of B19 neurons after injections with Sp-cAMP and Rp-cAMP

(A) B19 neurites following acute injection with 100 μM Sp-cAMP. Note the increased branching of neurites.

(B) Similar B19 neurites following injection with 100 μM Rp-cAMP, illustrating a decrease from control levels in the degree of branching.



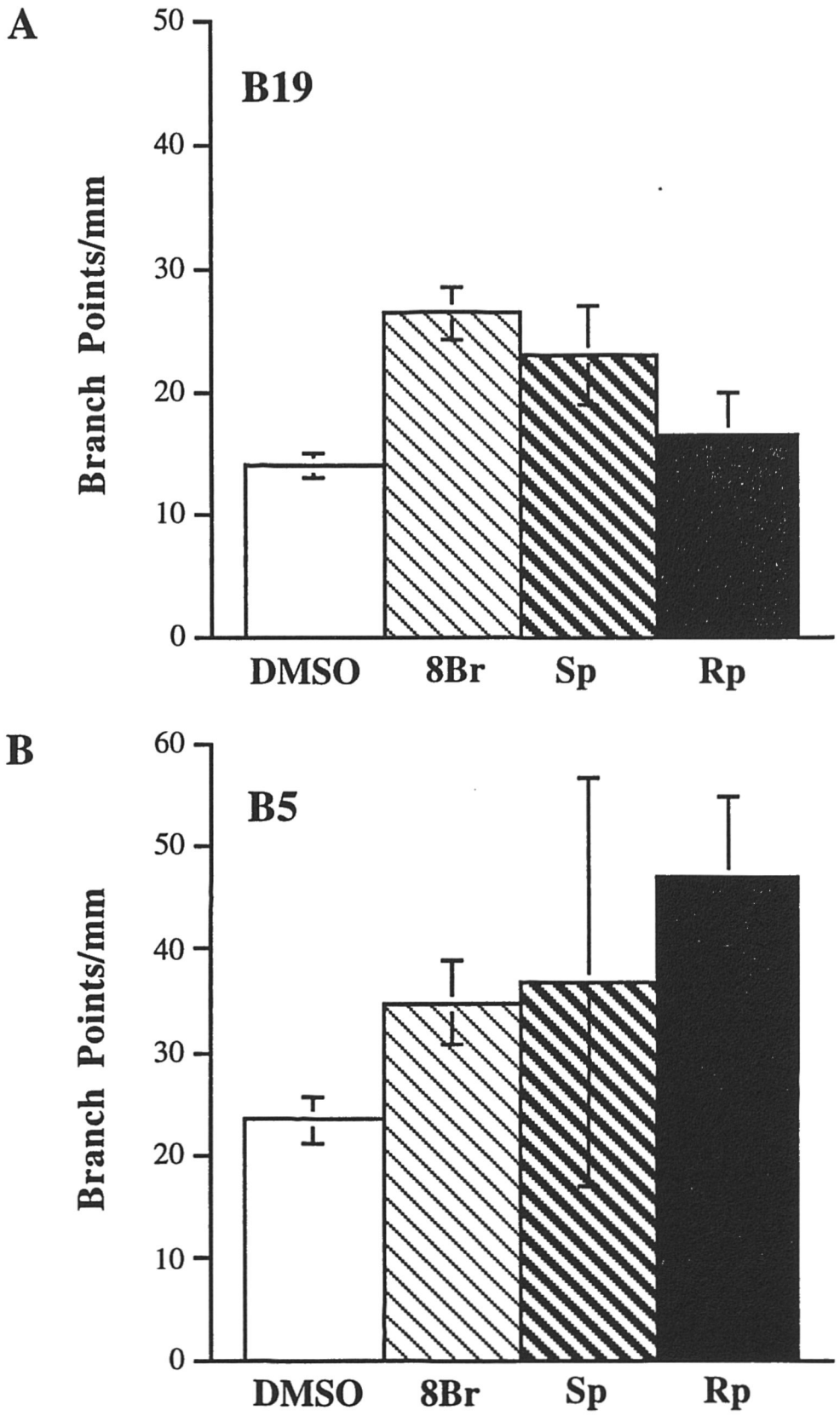


Fig. 5. Comparison of the degree of branching between neurons injected with DMSO and with 100 µM cAMP analog solutions.
(A) Branching comparison between DMSO-injected and cAMP-analog-injected B19 neurons.
(B) Branching comparison between DMSO-injected and cAMP-analog-injected B5 neurons. All values shown are in branch points per millimeter ± standard deviations.

Table 1. Experimental and Control Data Collected from B19 and B5 Neurons

Experimental Condition	n (number of cells studied)	number of original neurites	branch points per millimeter	varicosities per millimeter
B19				
DMSO control	15	7.4 ± 1.2 •	13.9 ± 0.9 •	6.7 ± 1.6 •
8-Br-cAMP	8	6.9 ± 0.7	26.3 ± 2.1^{TT}	10.2 ± 2.4
Sp-cAMP	10	7.5 ± 1.0	23.0 ± 4.0*	19.4 ± 4.1**
Rp-cAMP	8	7.4 ± 1.6	16.4 ± 3.5	30.8 ± 10.1**
B5				
DMSO control	8	14.0 ± 1.5	28.3 ± 2.2	23.1 ± 3.3
8Br-cAMP	6	17.5 ± 3.2	34.8 ± 4.1*	21.4 ± 1.7
Sp-cAMP	2	16.0 ± 8.5	36.8 ± 19.7	14.8 ± 1.3
Rp-cAMP	8	18.3 ± 1.6^{T}	46.9 ± 7.8**	29.0 ± 3.4^{T}

^{T} = $p<0.05$, comparison of analog vs. DMSO control

* = $p<0.02$, comparison of analog vs. DMSO control

** = $p<0.005$, comparison of analog vs. DMSO control

^{TT} = $p<0.0001$, comparison of analog vs. DMSO control

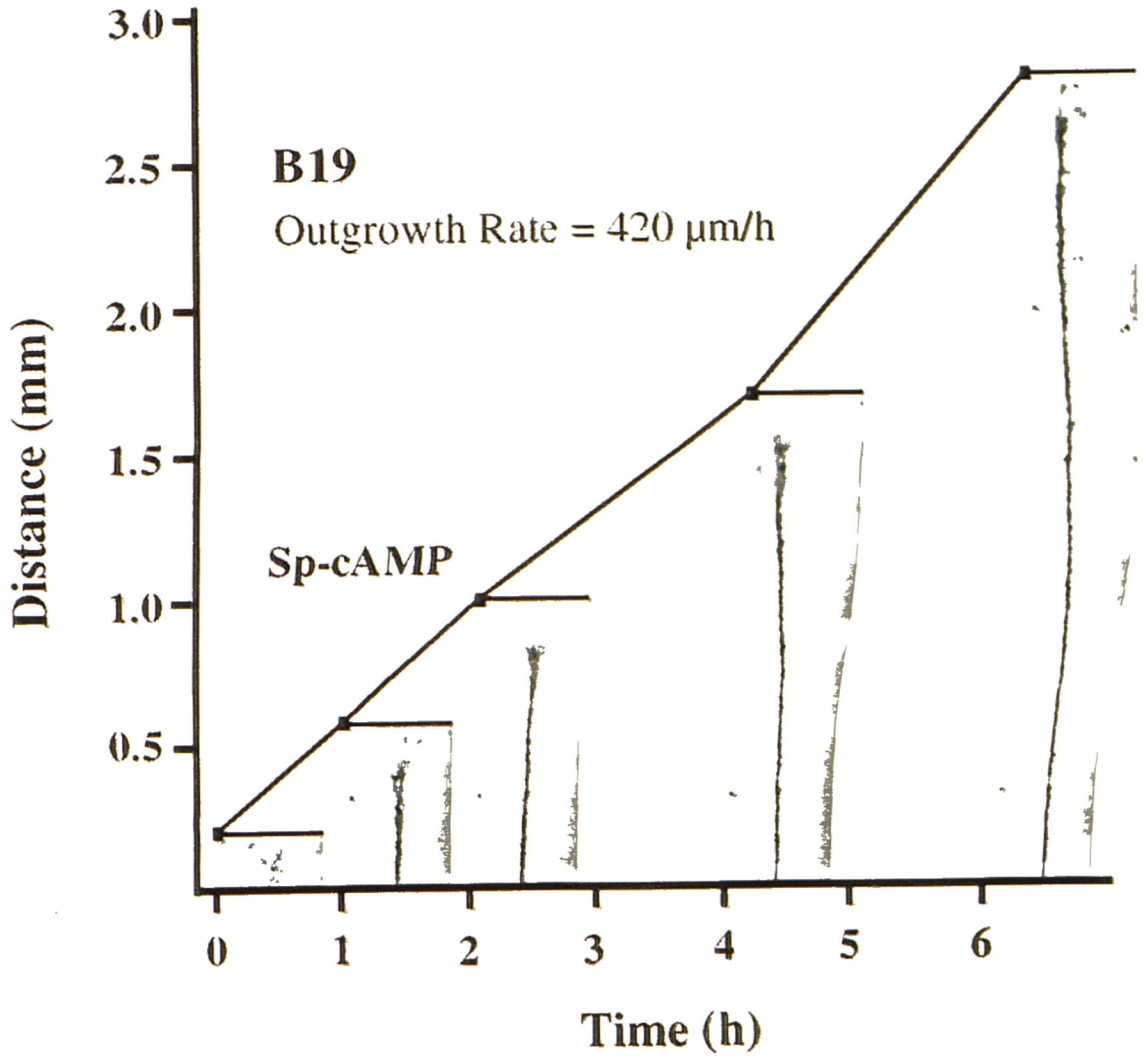
• = $p<0.01$, comparison of B19 control vs. B5 control

extension.

These studies also demonstrated that the degree of branching in B19 increased after injection with Sp-cAMP and 8Br-cAMP solutions (B5 neurons were not studied here). However, B19 neurons injected with Rp-cAMP showed no effect on branching after injection (Fig. 7). Branching observed in cells injected with a control solution was similar to the degree of branching expected in an actively-growing neurite of a non-injected cell.

A source of potential concern in these acute studies is that the cells were injected with analogs at the cell body, not at the growth cone; therefore, there could be a differential distribution of the propensity to branch depending on the diffusion patterns of the analogs throughout the neuritic arborizations. This effect was, of course, not a concern in the previous paradigm when injection was made prior to initial neurite outgrowth.

Fig. 6. Graphical representation of the effects over time of cellular injection of Sp-cAMP on the distance traveled by outgrowing B19 neurites. These B19 neurons were injected with Sp-cAMP at the time point indicated on the graph. Injection of Sp-cAMP had little, if any, effect on the growth rate of neuron B19.


B19
Outgrowth Rate = 420 µm/h
Sp-cAMP
Distance (mm)
3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
Time (h)

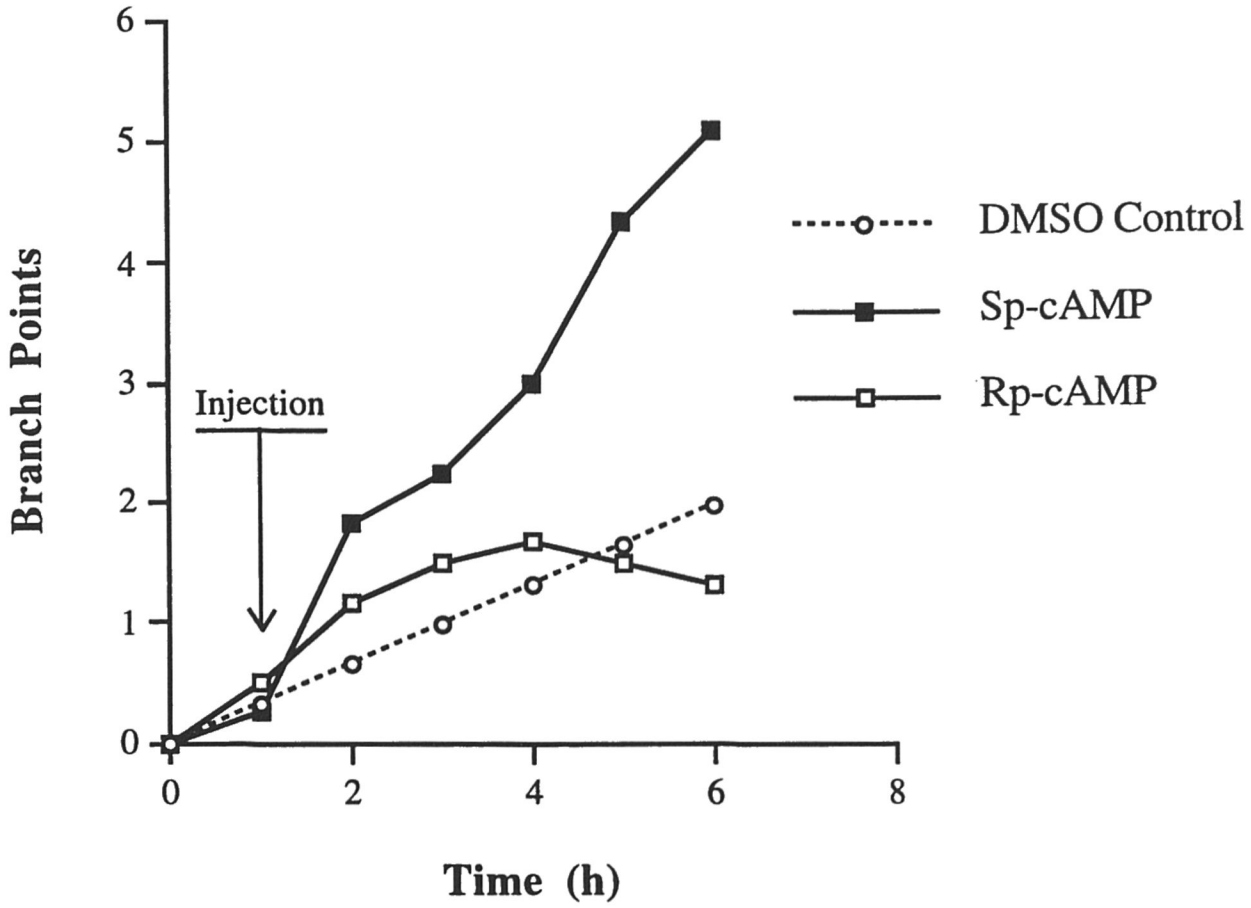
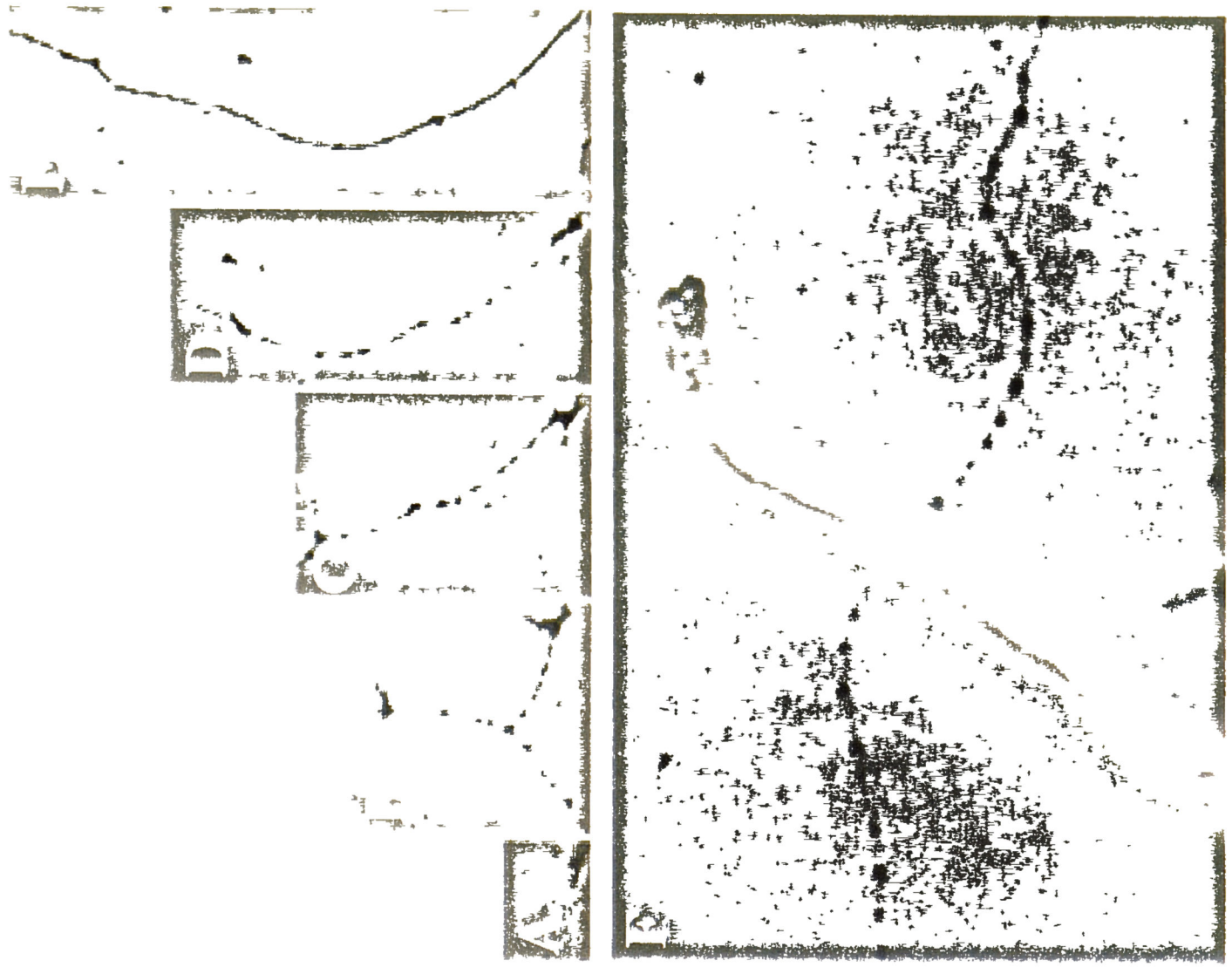


Fig. 7. Representation of the effects of cell-body injection with Sp-cAMP and Rp-cAMP on the degree of branching (branch points/mm) over time (hours post-plating). The dotted line on this graph represents the linearity of extension in neurons injected with DMSO solution. Sp-cAMP-injected neurons exhibited a marked increase from the control rate in the degree of branching after injection, whereas Rp-cAMP-injected cells displayed a slight decrease in the degree of branching over time.

Additional Observations of Growth Cone Behavior

In addition to alterations seen in branching patterns, varicosities, and the rates of neurite elongation, we observed several other interesting growth cone behaviors. First, as indicated in Figure 7, we observed a subtle decrease in branching after injection of Rp-cAMP. This finding correlates with a retraction of previously formed branches in these injected cells (Fig. 8A). Secondly, after the injection of Sp-cAMP to B19 neurons, evidence of neuritic defasciculation was observed in several neurons (data not shown). This observation is particularly intriguing since recent work in Drosophila has suggested a correlation between fasciculation/defasciculation states and cAMP levels (Schuster et al., 1996). Finally, after injection of Rp-cAMP, there was not an increase in neuritic branching following neuron-muscle interactions. This is interesting since previous work has demonstrated that B19 possessed increased branching frequency following muscle contact in untreated cultures (Zoran et al., 1996). Although there was no evidence of this target-induced branching behavior (mean branch points/mm = 10.7 4.3; n=5) after injections with Rp-cAMP, there was an increased frequency of varicosities noticed after Rp-cAMP injections (varicosities/mm = 59.9 24.3; n=5) (Fig. 8B).

Fig. 8. **(A-E)** Illustration of the retraction of branches from outgrowing B19 neurites following Rp-cAMP injection. **(F)** The effects of neuron-muscle contact on B19 neurites following Rp-cAMP injection. After neuron-muscle contact, the incidence of varicosities is markedly increased, and inhibition of neuritic branching is observed.



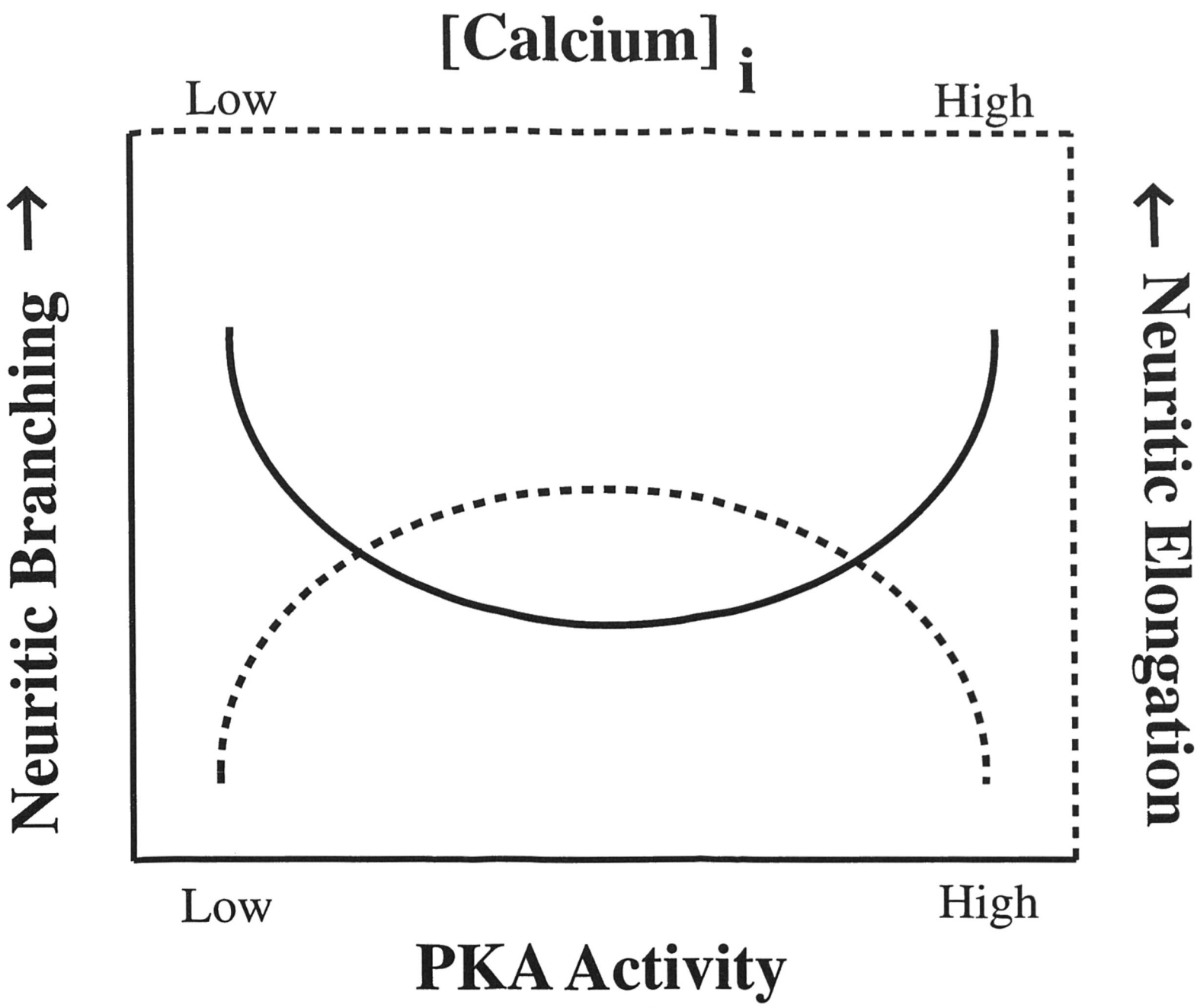


Fig. 9. Proposed hypothetical model of the relationship between cyclic-AMP-dependent protein kinase A (PKA) activity, intracellular calcium concentration, and rates of neuritic growth and branching. At moderate levels of PKA activity and moderate calcium concentrations, outgrowth of neurons is within an optimal range. This model suggests that low or high calcium concentrations and low or high levels of PKA activity cause branching to increase while growth is inhibited.

DISCUSSION

These cell culture studies of the effects of cyclic-AMP analogs on identified *Helisoma* neurons have demonstrated that branching of a neuronal process is affected by the activity of cyclic-AMP-dependent protein kinase A (PKA). Several specific lines of evidence indicate that this is indeed correct. First, injection of 8-bromo-cAMP caused a significant increase in the probability of branching in B19 at 48 hours post-injection. Second, injection of a second analog, Sp-cAMP, showed a significant increase in the probability of branching at 48 hours after injection. Third, the analog Rp-cAMP, which decreases PKA activity, had no effect on branching in this paradigm. Finally, data acquired from acute injections into neurite-bearing cells established that the injection of Sp-cAMP into cells with existing neurites and growth cones still increased the probability of branching and injection of Rp-cAMP in fact resulted in a decreased probability of branching. These later observations indicated that results of the chronic paradigm were not due to analog effects on initiation of outgrowth, but rather were effects of branching. A caveat to this argument, however, involves the site of chemical injection. In both experiments, injections were made at the cell body and not at the growth cone. This means that the diffusion of the injected chemical could have been dispersed differentially throughout the growing neurites, so that it would not be possible to distinguish which neurites were actually affected by analog injection. This would not be a concern in chronic studies where injections were made prior to neurite outgrowth. Taken together, these results suggest that changes in the activity of cyclic-AMP-dependent PKA elicit subsequent changes in the probability that a neurite will branch.

Previous studies have demonstrated that growth cones of specific *Helisoma* neurons respond differently following exposure to chemical signals such as neurotransmitters (Haydon et al., 1986; Murrain et al., 1990). Similarly, we have detected differences in the effects of cAMP analogs on the branching of these specific neurons, B5

and B19. The basal likelihood for a control neuron to branch (i.e., the initial branching set-point), differed between B5 and B19. Basal branching levels in B5 was almost twice that of B19, approximately 23 branches per millimeter in B5 versus 14 branches per millimeter in B19. What cellular mechanisms contribute to these cell-specific branching rates remain unclear and it is also not known what the set-point levels of PKA are in either type of neuron. Thus, elevating PKA levels in one cell may mean changing it from low to moderate activity levels, whereas in another cell, it might mean changing levels from moderate to high. We have also observed that there are higher basal levels of both original neurites and the number of varicosities in B5 than in B19 control neurons. This fact may support the notion that set-points of a variety of neuronal characteristics differ between these two cells. On the other hand, these results may be indicative of a complex relationship between PKA activity in a growth cone and changes in neuronal architecture. Such dynamic control of growth cone behavior has been suggested by studies of cultured neurons from *Drosophila* memory mutants with altered cAMP levels (Kim and Wu, 1996). These studies indicate that growth cone motility may require optimal cAMP levels within an operational range.

Interestingly, there are also significant increases in branch points in neurons injected with Rp-cAMP. Neuron B5 showed that both increased and decreased cyclic-AMP levels resulted in significant increases in branching. This result seems to be paradoxical because one might expect Rp-cAMP, an inactivator of PKA, to have the opposite effects of Sp-cAMP and decrease the levels of branching in a given neurite.

A similar model for such dynamic PKA-dependent control of neuronal branching is presented in Figure 9. This model also suggests an operational range of PKA activity levels in growing neurites. An inverse model has been proposed for calcium regulation of neurite outgrowth in these cells. This calcium model hypothesizes a range of permissive intracellular calcium concentrations over which a neurite will exhibit elongation. Concentrations above and below this region would elicit a decrease in growth cone motility

and outgrowth (Kater and Mills, 1991). By extension, our model hypothesizes that PKA activity, at moderate levels, is restrictive to neuritic branching, but activity levels above or below this restrictive range would be permissive to branching (Fig. 9). Thus, this model attempts to explain a complex inverse relationship where levels of PKA activity and intracellular calcium might regulate the propensity for a growth cone to branch and/or elongate.

How might such a regulatory mechanism be important to neuronal development and synapse formation? The data and model presented in this thesis suggest an inverse relationship between the rate of neuritic elongation and the probability of branching. This is important because it has been shown that cyclic-AMP-dependent alterations in intracellular calcium levels occur when neurites encounter specific targets (Zoran et.al., 1993; Funte and Haydon, 1993; Zoran and Poyer, 1996). In addition, James Poyer, a collaborator in the lab, conducted studies using the same neurons used for image analysis in the present studies. In these electrophysiological recordings, he investigated the levels of synaptic transmission in the various analog-injected cells. Interestingly, he found that Sp-cAMP injections caused significant increases in B19 synaptic function, but injections with Rp-cAMP did not (J. Poyer, personal communication). Therefore, it seems that the activity of PKA in a B19 neuron has profound effects on the synaptic capacity of its neurites. One can imagine that combinations of various environmental cues affecting PKA activity could have multiple effects on neuritic elongation, branching, and/or development of synaptic transmission. These integrated cellular modifications following target contact might culminate in decisions of synaptic specificity based on alterations in growth cone behavior and subsequent modifications in neuronal architecture.

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