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Calcium Regulation of Neurite Elongation and Growth Cone Motility

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Neurite outgrowth from isolated, identified molluscan (*Helisoma trivolvis*) neurons in culture can be suppressed by neurotransmitters and electrical activity, both of which increase intraneuronal Ca^{2+} levels (Haydon et al., 1984; Cohan et al., 1986, 1987). We explored the possibility of a causal relationship between Ca^{2+} influx from the cell exterior and neurite outgrowth using a spectrum of pharmacological manipulations known to affect transmembrane Ca^{2+} flux. Ca^{2+} ionophore A23187, an agent expected to increase Ca^{2+} influx, suppressed both elongation and motile growth cone structures (i.e., filopodia and lamellipodia) in a dose-dependent (10^{-8}–10^{-6} M) and reversible manner. Furthermore, high concentrations of Ca^{2+} channel blockers (La^{3+}, Cd^{2+}, Co^{2+}; e.g., 10^{-4} M La^{3+}) suppressed both elongation and growth cone movements. These data support previous experiments, which indicated that neurite outgrowth is dependent upon a specific range of intracellular Ca^{2+} concentrations (Connor, 1986; Cohan et al., 1987). However, tests of the dose-dependency of the effects of Ca^{2+} channel blockers on outgrowth revealed that specific, low concentrations of Ca^{2+} channel blockers (e.g., 10^{-5} M La^{3+}) caused, simultaneously, a reduction of growth cone filopodia and an acceleration of elongation. Consistent with the results using low levels of Ca^{2+} channel blockers, reduced extracellular Ca^{2+}-stimulated neurite elongation while suppressing growth cone motility. Finally, neurotransmitter regulation of neurite outgrowth was shown to require influx of extracellular Ca^{2+}; serotonin inhibition of neuron B19 was prevented by La^{3+} (10^{-5} M) or by incubation in a reduced Ca^{2+} environment. Taken together, these results indicate that there are optimum levels of Ca^{2+} influx that promote normal neurite elongation and growth cone movements; these 2 components of outgrowth appear to have differential sensitivities to Ca^{2+}.

The biochemical basis of neuronal outgrowth has been an area of intense investigation. The pioneering work on nerve growth factor led the way in uncovering a variety of environmental conditions and specific molecules that regulate the initiation of neuronal outgrowth (e.g., Levi-Montalcini and Angeletti, 1968; Schubert et al., 1978; Greene and Shooter, 1980; Gundersen and Barrett, 1980). The cellular mechanisms of action of the regulators of neuronal outgrowth are not clear, but second messengers, including cAMP (Schubert et al., 1978; Nirenberg et al., 1984), calcium (Schubert et al., 1978; Connor, 1986; Cohan et al., 1986, 1987), and inositol phospholipids (Ishii, 1978; Spinelli and Ishii, 1983; Hama et al., 1986) are implicated. Ca^{2+} affects the assembly states of both microtubules (Schliwa et al., 1981) and microfilaments (Adelstein and Eisenberg, 1980) and is a prime candidate as an intracellular regulator of neurite elongation and motile growth cone structures (filopodia and lamellipodia).

In understanding the genesis of functional neuronal architecture, the regulation of cessation of neuronal outgrowth is perhaps of equal importance to the promotion of outgrowth. Little knowledge has been available on the specific chemical mediators that stabilize previously growing neurites. We recently described 2 independent signals that inhibit neuronal outgrowth: neurotransmitters from exogenous sources (Haydon et al., 1984) and electrical activity (Cohan and Kater, 1986). These findings were possible, in part, because of the ability to culture isolated, identified neurons of the mollusk *Helisoma* (see Kater, 1985, for a review). The presence of specific neurotransmitters can profoundly alter neuronal outgrowth. Serotonin, for instance, can inhibit the neurite elongation and growth cone movements of particular identified neurons, while not affecting other specific neurons (Haydon et al., 1984). While neurotransmitters act on defined subsets of neurons, we have found that a second regulatory variable, the generation of action potentials, acts on all tested neurons to abruptly terminate filopodial and lamellipodial activities and neurite elongation (Cohan and Kater, 1986).

Several authors have suggested a role for Ca^{2+} in the regulation of neurite outgrowth (Schubert et al., 1978; Llinas, 1979; Gundersen and Barrett, 1980; Anglister et al., 1982; Kostenko et al., 1983; Bixby and Spitzer, 1984), and this second messenger system was thus an obvious candidate for direct measurement. Connor's (1986) initial fura-2 studies on cultured mammalian CNS neurons gave the first substantive data on intracellular Ca^{2+} and outgrowth. Cohan et al. (1986, 1987) were then able to measure intracellular Ca^{2+} in neurons exposed to substances and conditions known to affect outgrowth (serotonin or induced action potentials); they demonstrated strong correlations between the level of free cytoplasmic Ca^{2+} and the growth status of the neuron. Their data indicated that an optimal level of free intracellular Ca^{2+} was required for outgrowth to continue.

The present study further tests a "calcium hypothesis" of control of neurite outgrowth by direct manipulation of Ca^{2+} flux across the plasma membrane. Standard pharmacological agents known for their particular effects on Ca^{2+} were employed to help establish a causal relationship between Ca^{2+} influx from the cell

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exterior and neurite outgrowth. Since the pharmacology of Ca^{2+} can be capricious, we used a spectrum of agents and found quite consistent results using Ca^{2+} ionophore A23187, Ca^{2+} channel blockers (La^{3+}, Co^{2+}, and Cd^{2+}), and reduced or zero Ca^{2+} medium. The combined results of the experiments reported here demonstrate that motile growth cone structures, as well as neurite elongation, are regulated by Ca^{2+} influx. However, the optimal levels of Ca^{2+} influx required by these 2 components of outgrowth appear to differ, a finding that allows further definition of the roles of growth cones in the generation of neuronal architecture.

Materials and Methods

Animal dissection and neuronal culture. Adult snails of *Helisoma trivolvis* (red) with shell diameters of 10–18 mm were maintained as previously described (Haydon et al., 1985). Procedures for dissections, buccal ganglia removal, and identified neuron isolation have been described previously (Wong et al., 1981; Haydon et al., 1985). Isolated neurons were transferred to culture dishes with a polylysine-coated glass or plastic substratum, containing 2 ml of defined HL-15 medium (Gibco, Grand Island, NY) conditioned with *Helisoma* brains [conditioned medium contains a factor that associates with the substratum and promotes extensive neurite outgrowth (Wong et al., 1981, 1984)]. Neurons were cultured in a humidified room air atmosphere at 22–25°C.

Treatment of neuronal cultures. All experiments were conducted on growing identified buccal neurons B19 and B5 at 20–24 hr postplating. Ionophore A23187, nifedipine, and verapamil were dissolved in dimethyl sulfoxide (DMSO) and were added to cultures in 5–10 μl volumes (DMSO alone at these levels was added to control plates and did not affect outgrowth parameters). $LaCl_3$, $CoCl_2$, $CdCl_2$, sodium dantrolene, and serotonin were dissolved in HL-15 and added to cultures in 20–200 μl volumes. All concentrations reported are the final concentrations in culture medium. All agents except sodium dantrolene (Norwich Eaton Pharmaceuticals, Norwich, NY) were from Sigma Chemical Co. (St. Louis, MO). Reduced Ca^{2+} medium consisted of HL-15 in which $CaCl_2$ was replaced with $MgCl_2$; this medium likely contains at least 200 μM Ca^{2+}, originally present as contamination (Raaflaub, 1956). Zero Ca^{2+} medium consisted of HL-15 in which $CaCl_2$ was replaced by $MgCl_2$ with added 1 mM EGTA. Cells were incubated in a room air atmosphere at 22–25°C. Medium changes were performed by carefully removing all but a thin film of medium and then gently adding fresh medium to the dishes. Growth cones were isolated from neurons with the aid of an electrolytically sharpened tungsten microknife; neurites were cut just proximal to the growth cone.

Assessments of neurite elongation rate and growth cone morphology. Cells and isolated neurites were examined and photographed on a Nikon Diaphot inverted microscope with phase-contrast optics. Two parameters of neurite outgrowth were assessed: neurite elongation and growth cone structures associated with motility (filopodia). Neurite elongation rates were quantified using tracings made from projected negatives of photographs taken sequentially. Linear-regression analysis of pre- and posttreatment elongation rates was used to obtain the post/pretreatment ratio, which is expressed as the percentage of the initial elongation rate. Growth cones were also examined on the projected photographic negatives; the number of filopodia/growth cone was used as index of motility since these structures are known to reflect the underlying activity of the growth cone cytoskeleton (Bray and Gilbert, 1981). Student's *t* test was used for all statistical comparisons, and values are expressed as mean and SEM.

Results

Effects of elevations or reductions in Ca^{2+} influx on neurite elongation and growth cone morphology

Previous measurements of intracellular Ca^{2+} using fura-2 indicated that the intracellular Ca^{2+} level is maintained within a narrow range that is optimal for outgrowth; outgrowth was suppressed in neurons in which intracellular Ca^{2+} was increased by exposure to neurotransmitters or electrical activity, while neurons that stopped growing spontaneously had significantly lower levels of Ca^{2+} within their neurite tips (Cohan et al., 1986, 1987).

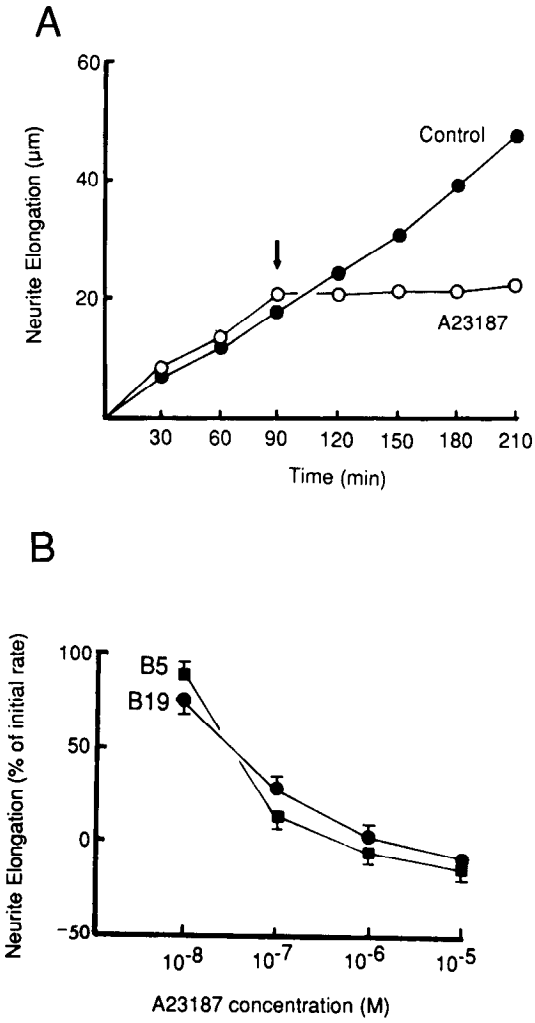

Figure 1. Time course and dose-dependence for inhibition of neurite elongation by A23187. *A,* Representative time course of A23187 suppression of neurite elongation. Neurons B5 and B19 were preincubated for 90 min, followed by a 2 hr incubation in the absence (*Control*) or presence of 10^{-7} M A23187 (*arrow,* addition of A23187). Examples shown are representative of all 33 neurites assessed. *B,* Following a 90 min preincubation to determine the initial rate of neurite elongation, neurons B19 and B5 were incubated 120 min in the presence of the indicated concentrations of ionophore A23187. Values are expressed as percentages of the initial elongation rate and represent the means and SEM of determinations on 11–22 neurites from 3–6 neurons. Values for A23187 at concentrations of 10^{-7} M or greater were significantly different from control values ($p < 0.01$–0.0001), which averaged 101.0 ± 4.6% of the initial rate.

In all cases in which neurite outgrowth was suppressed, the neurite tips were devoid of filopodia and lamellipodia, suggesting that growth cone movements and neurite elongation are associated processes. To causally relate the degree of Ca^{2+} influx from the cell exterior to changes in neurite elongation and growth cone movements, we assessed outgrowth after exposing neurons to manipulations known either to increase (A23187; Reed and Lardy, 1972) or reduce (Ca^{2+} channel blockers; Weiss, 1974; Hagiwara and Byerly, 1981; reduced or zero Ca^{2+} medium) Ca^{2+} influx. Further, we titrated these treatments to determine whether the elongation and growth cone movement components of outgrowth had differential sensitivities to Ca^{2+}.

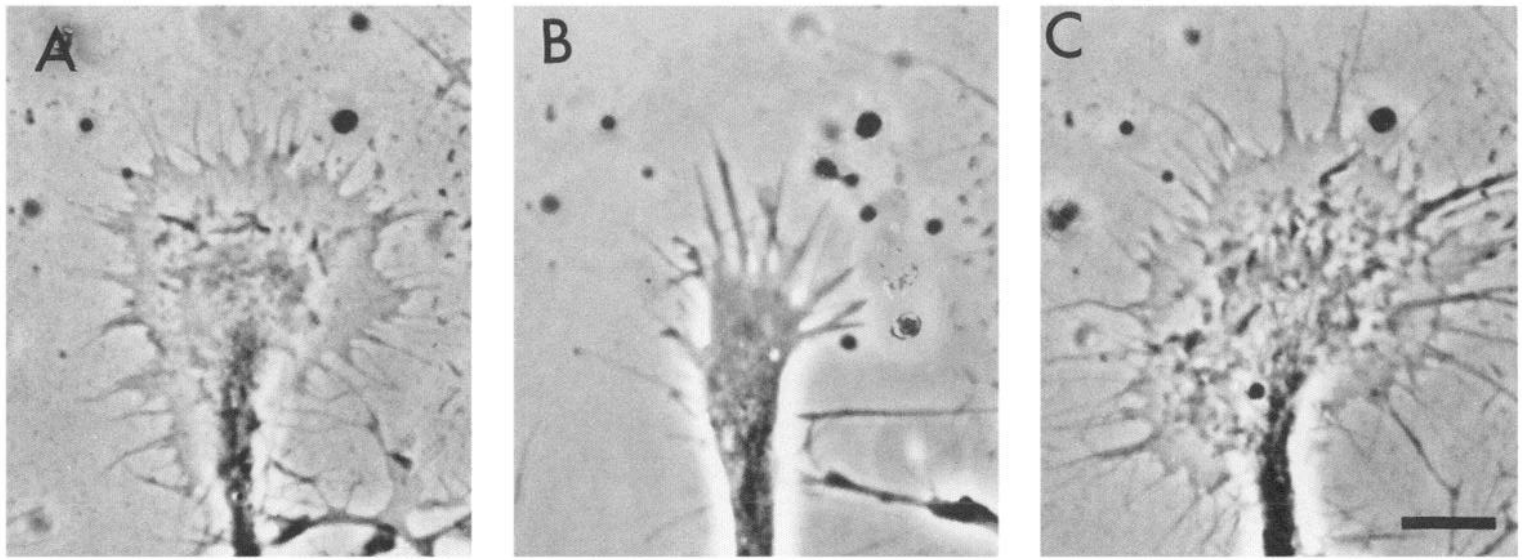

Figure 2. Reversibility of the effect of A23187 on neurite elongation and growth cone morphology. Following a 90 min preincubation to determine initial rates of neurite elongation, neurons B5 and B19 were exposed to ionophore A23187 (10^{-7} M) for 60 min, then washed (four 5 min washes) in fresh medium without A23187; incubations were then continued for an additional 120 min. Photographs depict the reversible suppressive effect of A23187 on the motile behaviors of a growth cone of a neuron B5: *A,* Immediately prior to treatment with A23187. *B,* Sixty minutes posttreatment. *C,* Sixty minutes postwash. The *values* under the photographs represent the average rates of neurite elongation (means ± SEM; n = 11) expressed as percentages of the initial rate (18.2 ± 7.6 μm/hr). Calibration, 10 μm.

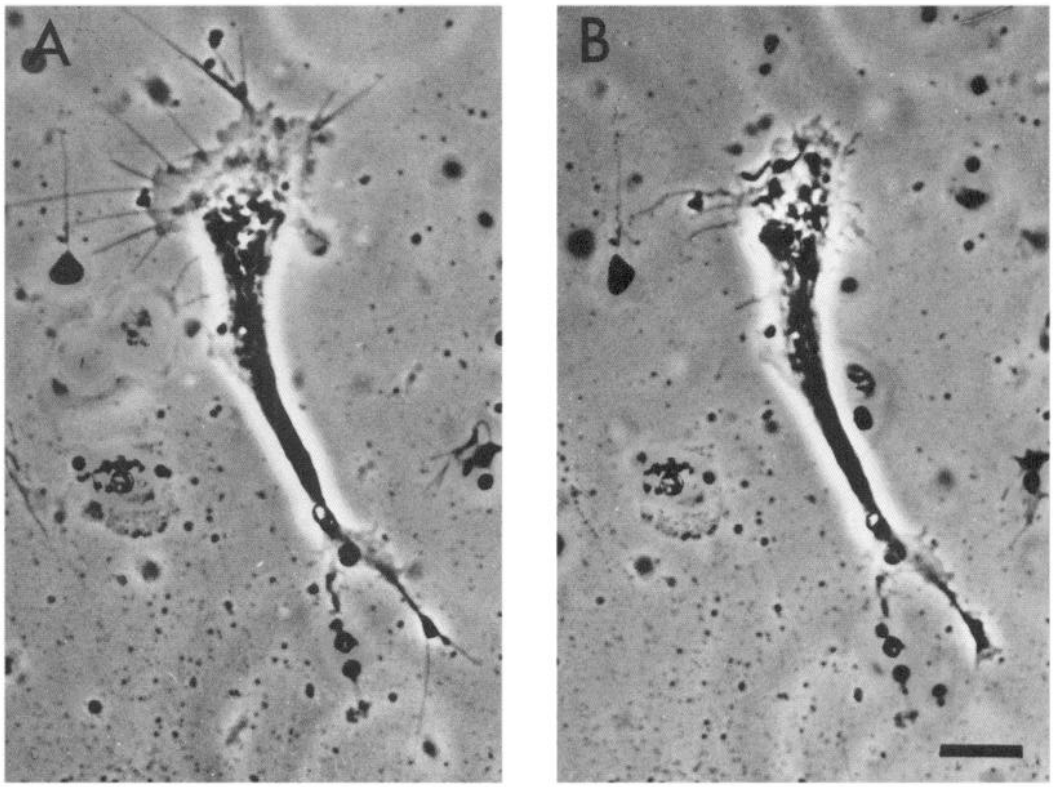

Figure 3. Effects of A23187 on the morphology of isolated growth cones. *A,* Photograph of an isolated growth cone from a neuron B5 taken 60 min after isolation and immediately prior to treatment with A23187; note the numerous filopodia and relatively broad lamellipodia. *B,* The same isolated growth cone as in *A,* 15 min following treatment with 10^{-7} M A23187; note the reduction in filopodial numbers and lamellipodial span. Calibration, 25 μm.

Effects of A23187 on neurite elongation and growth cone morphology. Neurite elongation was reduced to near zero within 30 min of treatment with A23187 at 10^{-7} M; the suppressive effect was dose-dependent for A23187 concentrations from 10^{-8} to 10^{-5} M (Fig. 1). Neurite retraction occurred at the highest A23187 concentration of 10^{-5} M. The ED_{50} for suppression was approximately 6×10^{-8} M. The suppressive effect of A23187 on neurite elongation was reversible; following suppression by 10^{-7} M A23187, neurons were washed with fresh medium, and the neurite elongation rate returned within 60 min to the pretreatment level (Fig. 2). This reversibility was not simply the result of the medium change; when A23187 was included in the postwash medium, elongation remained suppressed (cf. Fig. 6).

The expression of growth cone motility can be described morphologically. Motile growth cones are flat and protrude numerous filopodia (Fig. 2*A*). In contrast, nonmotile growth cones generally assume a rounded or clubbed appearance, and have greatly reduced numbers of filopodia (Fig. 2*B*). The suppression of elongation caused by A23187 at all effective concentrations was accompanied by retraction of growth cone filopodia and lamellipodia, resulting in a clubbed neurite tip (Fig. 2*B*) similar to that accompanying spontaneous, or neurotransmitter or electrically induced, cessation of outgrowth (Haydon et al., 1984; Cohan and Kater, 1986). A23187 caused significant reductions in the numbers of filopodia on growth cones of both neurons B19 and B5 (Table 1). These morphological changes were completely reversed following washout of the ionophore as growth cone filopodia and lamellipodia reappeared and elongation resumed (Fig. 2). The temporal relationship of reductions in growth cone motility and neurite elongation was such that elongation ceased within 30 min of exposure to A23187 and before all filopodia had retracted (Fig. 2). In most cases (8 of 10 neurites examined) no filopodia were evident by 90 min posttreatment.

To determine whether the suppression of growth cone movements caused by A23187 was exerted locally on the growth cones or required mediation by the soma, we tested the effects of A23187 on isolated growth cones. A23187 (10^{-7} M) stopped elongation of isolated growth cones (the rate was reduced to near zero in 11 of 11 isolated growth cones). Furthermore, A23187 caused the retraction of filopodia and lamellipodia in isolated growth cones (Fig. 3). Thus, increased Ca^{2+} influx can act locally to suppress both neurite elongation and growth cone movements.

Table 1. Effects of A23187 and La^{3+} upon growth cone filopodia

Treatment	Neuron	Filopodia/growth cone: Before treatment	Filopodia/growth cone: After treatment
A23187 (10^{-7} M)	B5	21.8 ± 2.3[a]	3.7 ± 0.9*
	B19	8.8 ± 0.6	0.8 ± 0.3*
La^{3+} (10^{-5} M)	B5	16.4 ± 1.5	1.2 ± 0.4*
	B19	5.7 ± 0.6	0.5 ± 0.2*

Values represent the means ± SEM of determinations on 10 growth cones. Filopodial counts were made immediately prior to treatment and 60 min after treatment.

[a] Growth cones of neurons B5 possess significantly more filopodia than do B19 growth cones ($p < 0.05$; cf. Haydon et al., 1985).

* $p < 0.001$, compared to before-treatment values.

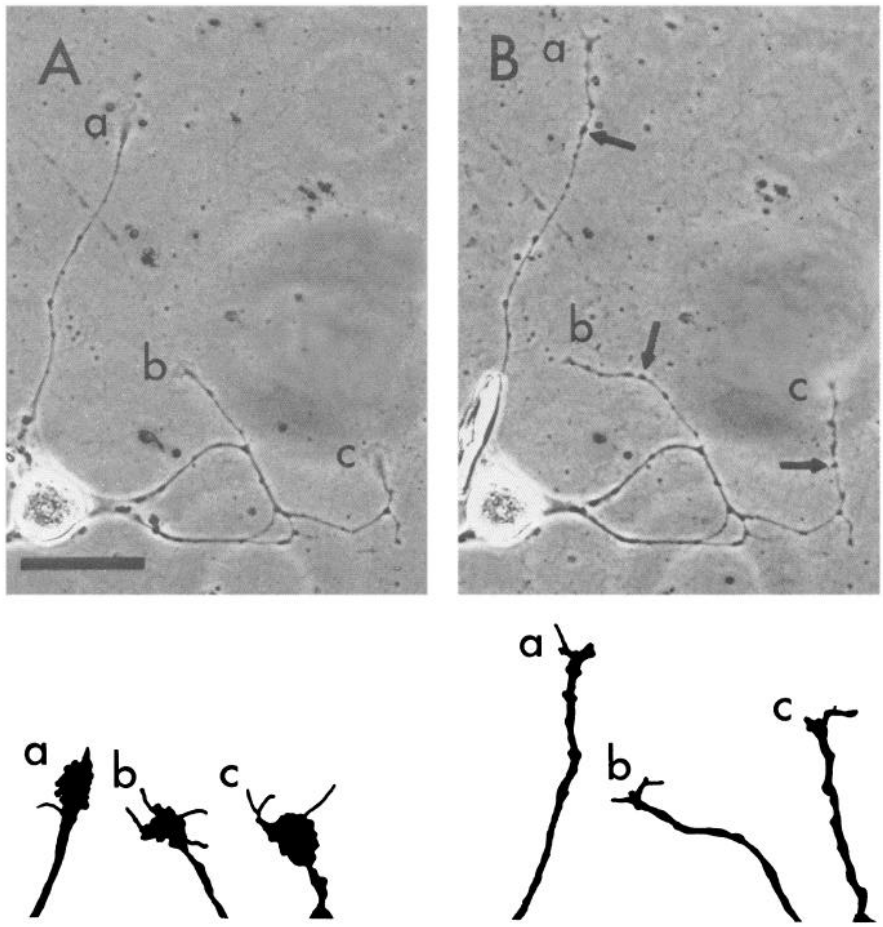

Figure 4. Effects of La^{3+} on growth cone morphology. *A*, A growing neuron B19 with active growth cones (*a–c*) immediately prior to exposure to $LaCl_3$ (final concentration, 10^{-5} M). *B*, The same neuron as in *A* 60 min after exposure to La^{3+}; growth cone filopodia and lamellipodia were reduced, but neurite elongation continued. *Arrows* point to varicosities present where growth cones existed prior to exposure to La^{3+}. *Drawings* represent enlarged tracings of the growth cones of the 3 neurites (*a–c*). The observations depicted by these photographs are representative of the morphological effects seen with La^{3+} on 35 of 37 neurites from 11 neurons. Calibration, 100 μm.

Effects of calcium channel blockers on neurite elongation and growth cone morphology. We exposed neurons to polyvalent cation Ca^{2+} channel blockers known to act on a broad spectrum of cell types (La^{3+}, Co^{2+}, Cd^{2+}; Weiss, 1974; Hagiwara and Byerly, 1981) and determined the dose-dependencies and specificities of their effects on neurite outgrowth. Consistent with the hypothesis that specific intracellular Ca^{2+} levels are optimal for normal outgrowth, we found that La^{3+} (10^{-4} M), Co^{2+} (10^{-2} M), and Cd^{2+} (10^{-4} M) all significantly suppressed neurite elongation (suppression to 51, 42, and 30% of control levels for La^{3+}, Co^{2+}, and Cd^{2+}, respectively; $p < 0.001$–0.0001; $n = 8$–20). This suppression presumably resulted from a reduction in Ca^{2+} influx and possibly from a lowering of intracellular Ca^{2+} levels. The retraction of growth cone filopodia and lamellipodia was coincident with suppression of elongation. Organic blockers of Ca^{2+} influx from the cell exterior (nifedipine and verapamil; Hagiwara and Byerly, 1981) and a blocker of release from intracellular stores (dantrolene; Van Winkle, 1976) were without effect at concentrations expected from other systems to block calcium fluxes (12–19 neurites, assessed from 3–5 neurons/treatment). This set of results further supports the hypothesis that neurite outgrowth is promoted only when Ca^{2+} influx is at a specific level required for outgrowth. However, further experiments, described below, indicate that the elongation and growth cone movement components of outgrowth appear to have somewhat different Ca^{2+} sensitivities.

Previous studies using cytoskeleton-altering drugs showed that elongation can occur in neurites devoid of filopodial and lamellipodial growth cone movements (Marsh and Letourneau, 1984; Letourneau, 1985). We observed a similar phenomenon when neurons were treated with lower concentrations of Ca^{2+} channel blockers than were used above (La^{3+}, 10^{-5} M; Co^{2+}, 10^{-3} M; Cd^{2+}, 5×10^{-5} M; Fig. 4). At these concentrations growth cone filopodia were significantly reduced (Table 1), but neurite elongation continued and was, in fact, accelerated (elongation rate was increased to 153, 182, and 123% of controls for La^{3+}, Co^{2+}, and Cd^{2+}, respectively; $p < 0.05$–0.001; $n = 9$–24). These low concentrations of Ca^{2+} blockers had similar effects on the elongation rates and morphology of isolated growth cones (not shown). Branching was not observed in neurites elongating in response to La^{3+} (35 neurites monitored for 4 hr following exposure to La^{3+}), while, under the same experimental protocol, nearly 50% (21 of 55) control neurites branched. Thus, given the appropriate status of cellular Ca^{2+}, neurites could elongate without filopodial growth cone movements, a condition that would greatly alter final neuronal architecture.

La^{3+} blocks serotonin suppression of neurite elongation

From 2 independent studies we knew that serotonin increases intracellular Ca^{2+}, on the one hand (Cohan et al., 1986, 1987), and suppresses neurite outgrowth on the other hand (Haydon et al., 1984). To determine whether serotonin inhibition is mediated by Ca^{2+} influx, we tested the effects of low levels of the Ca^{2+} channel blocker La^{3+} on neurite outgrowth from neurons B19 before and after exposure to serotonin (Fig. 5). Prior application of La^{3+} (10^{-5} M) prevented the normal suppressive

response of the neurotransmitter and, as reported above, significantly accelerated neurite outgrowth to 150–180% of the base rate ($p < 0.05$–0.01). Of major interest in the regulation of neurite outgrowth is the degree to which neurons that have stopped growing can resume neurite outgrowth. Neurites inhibited by serotonin could be induced to grow again by exposure to La^{3+}. In each of the 10 cases examined, neurites whose elongation and growth cone motility were completely suppressed by serotonin recovered and renewed elongation, though this was not true of growth cone motility following exposure to La^{3+} (Fig. 5). The morphology of these disinhibited neurites was indistinguishable from that seen for neurons treated with La^{3+} alone (compare Figs. 4 and 5); filopodial and lamellipodial structures were not observed in growth cones of La^{3+}-treated neurons, and the neurite tips extended as narrow (neurite-width) processes (Fig. 5).

Effects of reduced and zero extracellular calcium on neurite outgrowth: Interactions with serotonin, A23187, and La^{3+}

As a further test of the basic hypothesis of this study, we exposed neurons to media with either reduced ($MgCl_2$ substituted for $CaCl_2$) or zero ($MgCl_2$ substituted for $CaCl_2$ plus EGTA) Ca^{2+} and determined neurite elongation rates in the absence (controls) or presence of serotonin, A23187, or low levels of La^{3+}. Before performing these experiments, it was necessary to determine several baseline data. Previous studies indicated that medium changes can accelerate neurite outgrowth (D. P. McCobb, personal communication); we therefore used as controls neurons that were washed in fresh HL-15 of normal Ca^{2+} composition. As expected, washing significantly increased the rate of neurite elongation to what we designate as the "control" level, which was 140 ± 11% of the base rate ($p < 0.005$; Fig. 6). The medium change had no obvious effects on growth cone lamellipodial or filopodial movements. Incubation in reduced Ca^{2+} medium caused a reduction in growth cone filopodia and lamellipodia (not shown) and increased the rate of neurite elongation to 140% of controls ($p < 0.05$; Fig. 6). Using this same paradigm, it was found that in normal Ca^{2+} medium, La^{3+} significantly stimulated elongation to 160% of controls, while A23187 and serotonin significantly suppressed elongation rates to 28 and 35% of controls, respectively (Fig. 6). With these data it was possible to directly test the extracellular Ca^{2+} requirement for the suppressive actions of serotonin and A23187. The key experiments were to pair treatments of serotonin or A23187 with the reduced extracellular calcium environment. In the normal Ca^{2+} environment, serotonin decreased the rate of neurite elongation to 28% of controls, while this same treatment in the reduced extracellular calcium environment had a significantly lesser suppressive effect (suppression to 70% of controls; Fig. 6). These results indicate that the rise in intracellular calcium recorded by fura-2 measurements (Cohan et al., 1986, 1987) was indeed derived from calcium influxes from the extracellular space, and show that the influx is necessary for inhibition of neurite outgrowth. Indeed, a repeat of this experiment with A23187, a facilitator of Ca^{2+} influx, gave precisely the same results: The usual inhibitory effect of the ionophore (suppression of elongation to 35% of control levels) was not seen in the reduced extracellular calcium environment (Fig. 6). Thus, agents that suppress neurite outgrowth require extracellular Ca^{2+}, presumably for influx.

The results to this point showed that expected increases (using A23187 treatment) or large decreases (using high concentrations of channel blockers) in cellular Ca^{2+} are suppressive to both neurite elongation and growth cone motility. Since decreasing extracellular Ca^{2+} or moderate blockage of Ca^{2+} influx with low concentrations of channel blockers can increase the rate of neurite elongation, it was of interest to test the effects on neurite elongation of these 2 treatments combined. Together, these treatments resulted in a significant reduction in the rate of elongation, compared to the rates for neurons exposed to either treatment alone (Fig. 6). These results are in line with the concentration-dependent effects of Ca^{2+} channel blockers on neurite elongation rates. Thus, this dual treatment likely reduced Ca^{2+} influx below the critical level that allows for maximal rates of neurite elongation. Furthermore, the latter result suggests that a basal level of Ca^{2+} "leak" into the cell is required for neurite outgrowth. In support of this interpretation are the results of a final experiment in which cultured neurons were exposed to a true zero Ca^{2+} medium (containing 1 mM EGTA). In this environment the neurite elongation rate was reduced to 32 ± 18.4% of the initial rate ($n = 7$).

Discussion

The present study has provided support for the hypothesis that changes in Ca^{2+} influx from the cell exterior can regulate 2 basic components of outgrowth, neurite elongation and growth cone movements. Furthermore, it appears that these 2 components have somewhat different Ca^{2+} requirements. It is important to emphasize that this hypothesis is based on evidence from 2 different approaches. Previous work strongly correlated Ca^{2+} levels within the growth cone with the growth status of the neuron (Cohan et al., 1986, 1987; Connor, 1986). The present study, on the other hand, examined the effects on neurite elongation and growth cone behavior of a battery of agents known to change transmembrane Ca^{2+} movements. Both lines of evidence produced results entirely consistent with the above hypothesis.

We interpret the results of this study as indicating that cellular Ca^{2+} movements can be regulated over a range physiologically relevant to neurite outgrowth. During the usual outgrowth seen in cell culture, the intracellular Ca^{2+} level is likely above that optimal for neurite elongation, but is at an optimum for growth cone filopodial and lamellipodial movements. Therefore, low concentrations of Ca^{2+} channel blockers that suppress growth cones but promote elongation probably decrease a constant "leak" Ca^{2+} current into the cell. In support of such a basal rate of Ca^{2+} influx are the data of Freeman et al. (1985), who used a vibrating probe to measure steady inward currents, thought to be Ca^{2+}, at the growth cones of cultured fish retinal ganglion cells. Thus, by regulating the degree of Ca^{2+} influx, the outgrowth status of the neuron can be precisely tuned.

Several sets of data now suggest that a critical concentration of intracellular Ca^{2+} is necessary for normal outgrowth of neurites: (1) Increases in growth cone Ca^{2+} induced by serotonin or electrical activity (Cohan et al., 1986, 1987) or by A23187 (Figs. 1–3) caused a reversible inhibition of both neurite elongation and growth cone filopodial movements. (2) High concentrations of Ca^{2+} channel blockers also suppressed outgrowth. (3) Lower concentrations of Ca^{2+} channel blockers (and by inference a critical Ca^{2+} influx) simultaneously reduced growth cone movements and enhanced neurite elongation. (4) Combined exposure to treatments that independently increase outgrowth rates (i.e., reduced extracellular Ca^{2+} and La^{3+}) did not enhance elongation rates (Fig. 6), indicating that a specific level of Ca^{2+} influx is

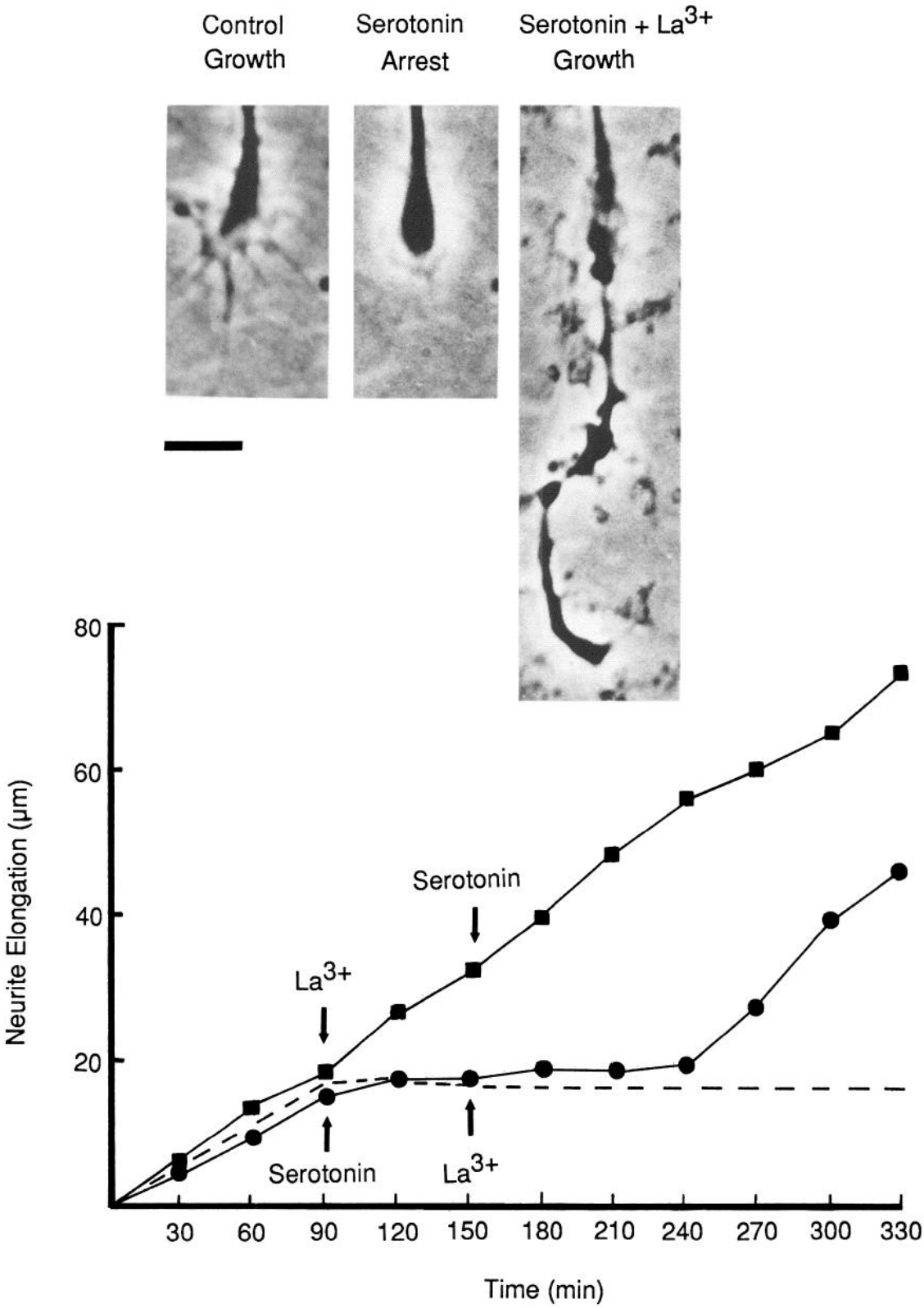

Figure 5. Effects of staggered La^{3+} and serotonin treatments on neurite elongation and growth cone morphology of neurons B19. Preincubation of 90 min was followed by addition of either La^{3+} or serotonin (10^{-5} and 10^{-4} M, respectively), as indicated by the *arrows*. The *dashed line* represents the average time course of elongation of neurites ($n = 7$) from a neuron B19 exposed to serotonin at the 90 min time point; note that suppression continues throughout the time course of the experiment. For combined treatments (*solid lines*), the points are from individual neurites and are representative of 5 (La^{3+} treatment followed by serotonin) or 10 (serotonin treatment followed by La^{3+}) cases. Statistical comparisons (values are expressed as percentages of the initial elongation rate, which averaged 11.8 ± 3.4 μm/hr) are as follows: For consecutive treatments with La^{3+} and serotonin, the rates were 155.2 ± 10.3% during exposure to La^{3+} prior to serotonin ($p < 0.005$ as compared to the initial rate) and 179.4 ± 15.9% following serotonin ($p < 0.005$ as compared to the initial rate). For consecutive treatments with serotonin and La^{3+}, the rates were 31.3 ± 5.9%

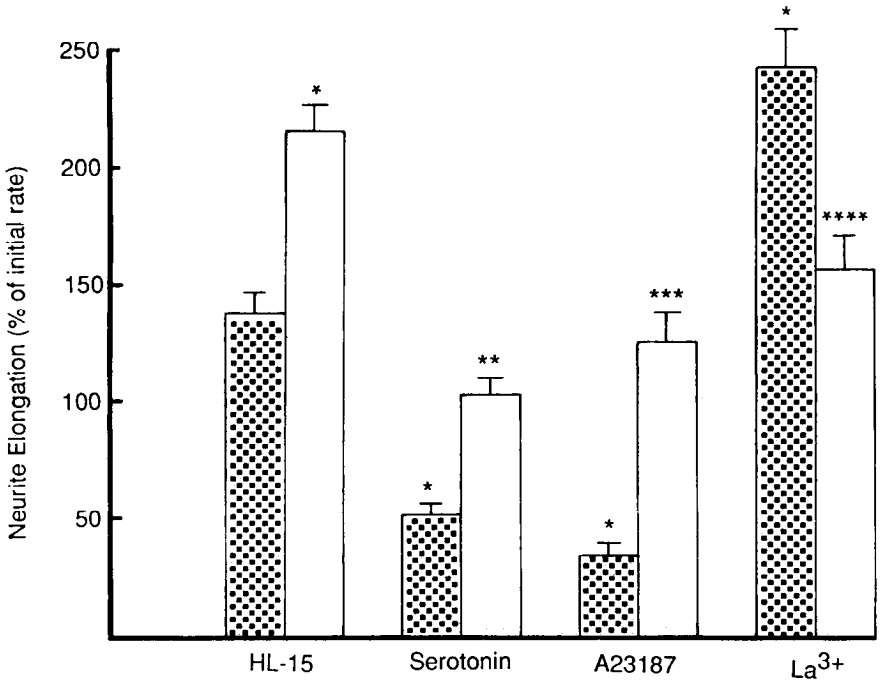

Figure 6. Effects on neurite elongation of serotonin, La^{3+}, and A23187 in relation to extracellular Ca^{2+}. Elongation rates of neurites from neurons B19 and B5 (serotonin experiments were performed on neurons B19) were determined before and after replacement of medium with fresh HL-15 containing either physiological levels of Ca^{2+} (4.1 mM $CaCl_2$, *stippled bars*) or reduced $CaCl_2$ HL-15 (*open bars*), with or without the following agents: serotonin (10^{-4} M), A23187 (10^{-7} M), or La^{3+} (10^{-5} M). Values represent the means and SEM of measurements on 11–18 neurites from 3–4 neurons/treatment group. *, $p < 0.01$–0.0001, as compared to HL-15, plus Ca^{2+}. **, $p < 0.01$, as compared to HL-15, minus Ca^{2+}; $p < 0.001$ as compared to serotonin, plus Ca^{2+}. ***, $p < 0.01$, compared to HL-15, minus Ca^{2+}; $p < 0.001$, as compared to A23187, plus Ca^{2+}. ****, $p < 0.05$, compared to HL-15, minus Ca^{2+}; $p < 0.05$, compared to La^{3+}, plus Ca^{2+}.

required for a maximum rate of outgrowth. Furthermore, the finding that removal of all extracellular Ca^{2+} with EGTA suppressed outgrowth indicates that some basal rate of Ca^{2+} movement into the neuron is required to maintain outgrowth (i.e., an equilibrium with cellular Ca^{2+} reduction mechanisms).

Although Ca^{2+} appears to be a general regulator of neurite outgrowth, it is also clear that responses depend on both the cell type studied and the experimental conditions employed. While previous investigators have used some of the agents we employed in the present study to test Ca^{2+} involvement in outgrowth regulation (Schubert et al., 1978; Gundersen and Barrett, 1980; Anglister et al., 1982; Kostenko et al., 1983; Bixby and Spitzer, 1984; Reboulleau, 1986), their studies did not use the full spectrum or the titrations of agents used here, and produced rather confounding and apparently contradictory results. For example, Reboulleau (1986) found that ionophore A23187 promoted neurite outgrowth in a rat CNS neuroblastoma cell line. While this result suggests that increases in Ca^{2+} influx promote outgrowth, our interpretation, based on the data of the present study, is that rates of Ca^{2+} influx prior to treatment in that particular system were below levels optimum for neurite outgrowth. Similarly, Gundersen and Barrett (1980) found that cultured chick dorsal root ganglion neurites turned towards a Ca^{2+} source when ionophore A23187 was included in the incubation medium. Quite different results were obtained in normal vertebrate (Letourneau and Wessells, 1974; Bixby and Spitzer, 1984) and molluscan (Kostenko et al., 1983; Cohan et al., 1987; and the present study) CNS neurons, where agents expected to increase Ca^{2+} influx inhibited neurite outgrowth. In contrast, in several neuronal tumor cell lines (Schubert et al., 1978; Anglister et al., 1982; Reboulleau, 1986) and in cultured chick retinal neurons (Suarez-Isla et al., 1984) Ca^{2+} influx promoted outgrowth. Of particular interest in relation to our data is the work by Bixby and Spitzer (1984), who reported a stimulation of neurite elongation rate in embryonic frog neurons by medium lacking Ca^{2+} and containing EGTA (growth cones were not examined). The latter result can be explained if one considers that Ca^{2+} influx rates in the cases of both frog embryonic and *Helisoma* neurons are above those optimal for neurite elongation, with the levels in the frog neurons likely being considerably above optimum than those in *Helisoma*.

The variety of results described above indicates that different neuronal types, examined under different environmental conditions, may have basal levels of intracellular Ca^{2+} either below, above, or within the range optimal for neurite elongation and/or growth cone movements. By considering the previous work in this context, it is possible to reconcile most of the observations made on Ca^{2+} and neurite outgrowth. This can be done by defining the basal level of intracellular Ca^{2+} in the neuron under study as the "Ca^{2+} set-point." For example, the Ca^{2+} set-point of *Helisoma* neurons in the present study was above the optimum level for neurite elongation but within the optimum range for growth cone movements. Thus, manipulations expected to increase Ca^{2+} influx (i.e., added A23187) suppressed both elongation and growth cone motility, while exposure to conditions expected to cause moderate reductions in calcium influx (low levels of Ca^{2+} channel blockers, reduced Ca^{2+} medium) suppressed growth cone motility and promoted neurite elongation.

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for the 2.5 hr interval following exposure to serotonin ($p < 0.001$, compared to the initial rate) and 155.6 ± 22.0% for the time points after 240 min ($p < 0.001$, compared to the rate during serotonin suppression). Micrographs are of a growth cone immediately prior to serotonin treatment (control growth), 60 min after exposure to 10^{-4} M serotonin and immediately prior to treatment with La^{3+} (serotonin arrest), and 3 hr after exposure to 10^{-5} M $LaCl_3$ (in the continued presence of serotonin). These effects were seen in all of 10 neurites from 6 neurons examined. Calibration, 25 μm.

Calcium channels and neurite outgrowth

A series of findings suggests that the effects on neurite outgrowth of multivalent cation blockers seen in *Helisoma* neurons are due to specific effects on Ca^{2+} channels: (1) We observed the following order of efficacy of Ca^{2+} blockers on neurite outgrowth: $La^{3+} > Cd^{2+} > Co^{2+}$, which was consistent with their order of potency in blocking Ca^{2+} currents and with their binding affinity in several invertebrate muscle and nerve preparations (Klee et al., 1973; Kostyuk et al., 1977). The lack of effect of organic blockers on neurite outgrowth is consistent with their relative lack of effect on calcium currents in other molluscan cells (Klee et al., 1973; Kostyuk et al., 1977); likewise the lack of effect of dantrolene, a blocker of calcium release from intracellular stores, on basal or serotonin-inhibited outgrowth is consistent with the plasma membrane Ca^{2+} channels being primary sites where serotonin acts to induce Ca^{2+} influx. (2) Co^{2+} blocked action potential-induced influx of Ca^{2+} into neurons B19 and B5 (Cohan et al., 1987). (3) Only a narrow concentration range of inorganic blockers induced neurite elongation. (4) Neurons incubated in reduced Ca^{2+} medium showed outgrowth behaviors (accelerated elongation and reduced growth cone movements) similar to those seen in neurons exposed to low levels of Ca^{2+} channel blockers. Furthermore, our findings indicate that a major locus of action of serotonin in increasing growth cone Ca^{2+} levels is at the entry of extracellular Ca^{2+}, rather than on release from intracellular stores. We found that under conditions of low levels of Ca^{2+} channel blockers or reduced extracellular Ca^{2+}, serotonin was less effective in inhibiting neurite outgrowth from responsive *Helisoma* neurons. It thus appears that serotonin acts to suppress outgrowth by activating intracellular events that ultimately open plasma membrane Ca^{2+} channels.

To fully understand how changes in influx result in changes in intracellular Ca^{2+}, we must also identify those mechanisms responsible for sequestering or buffering Ca^{2+}, as well as those for gating Ca^{2+} currents. In any case, it is clear from our data and from previous fura-2 studies (Cohan et al., 1986, 1987) that agents like serotonin induce increases in intracellular Ca^{2+} by increasing influx through the plasma membrane.

Calcium as a regulator of neuronal architecture

When and where a neurite branches during its development ultimately determines major aspects of neuronal form. In cell culture, where the process of branching is most readily studied, a branch point is seen as a region of splitting for an advancing growth cone. Thus, in the present study, under conditions expected to reduce Ca^{2+} influx, which allowed neurite elongation in the absence of growth cone filopodia and lamellipodia, it followed that branching failed to occur. These results are of obvious importance to the iteration of neuronal form and appear not to be an artifact of nonspecific effects of Ca^{2+} channel blockers or reduced Ca^{2+} medium. Similar effects have been observed in 2 other noteworthy cases. Vertebrate sympathetic neurons in culture were induced to grow in the absence of growth cone filopodia and lamellipodia; this was achieved by combined exposure to a microfilament-disrupting drug and an agent that promotes microtubule polymerization (Letourneau, 1985). Thus, elongation in the absence of growth cones is not a unique response of *Helisoma* neurons to low levels of Ca^{2+} channel blockers or reduced Ca^{2+} medium. In a study more directly related to normal neuronal development, LoPresti et al. (1973) reported that during the development of *Daphnia in situ,* only a few neuronal axons possessed growth cones, while others elongated without apparent growth cone structures. Taken together with these reports, our results lead to the conclusion that the cellular machineries underlying growth cone movements and neurite elongation have quite different roles in generating neuronal architecture. The filopodia and lamellipodia of growth cones are active in pathfinding and neurite branching, while other, relatively independent, cytoskeletal events associated with the neuritic shaft act to bring about elongation. Given this partitioning of roles and the differentiation of these 2 components of neurite outgrowth by treatments expected to influence influx of Ca^{2+}, it seems clear that neurons may regulate their form by carefully controlling cellular Ca^{2+} movements. A neuron may respond to environmental cues by growth, growth and branching, turning, or stopping by regulating Ca^{2+} movements within different regions of the neurite.

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