The Journal of Neuroscience, January 1992, 12(1): 96–103

Electrical Activity Increases Growth Cone Calcium but Fails to Inhibit Neurite Outgrowth from Rat Sympathetic Neurons

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Previous studies have shown that the growth of axons from both mouse dorsal root ganglion neurons and *Helisoma* neurons is arrested when the cells are electrically stimulated (Cohan and Kater, 1986; Fields et al., 1990a). Furthermore, in the case of *Helisoma* neurons, this arrest has been attributed to a rise in the calcium concentration in the growth cones (Cohan et al., 1987). To test the generality of these results, we examined the response of cultured rat superior cervical ganglion (SCG) neurons to electrical stimulation and changes in cytoplasmic calcium. Suprathreshold electrical stimulation of SCG neurons at 10 Hz by extracellular patch electrodes for periods of up to 1 hr had no measurable effect on their rate of growth. In agreement with previous studies, electrical stimulation was accompanied by a rise in the internal calcium concentration: when measured by the fluorescence of fura-2, growth cone calcium levels rose from about 100 nM to greater than 500 nM and then settled to a plateau value of about 350 nM. Despite this increase, however, growth of SCG neurons' processes continued.

Our results show that electrical activity is not a universal signal for neurons to stop growing and that a rise in internal calcium does not always arrest the migration of growth cones.

Developing neurons use many cues to reach their targets and form appropriate synaptic connections. For example, evidence has accumulated in recent years that neurons grow along chemical and possibly adhesive gradients (for reviews, see Bray and Hollenbeck, 1988; Dodd and Jessell, 1988; Jessell, 1988; Patterson, 1988). Previous work with cultured neurons suggested that their development might be regulated by electrical activity or precisely timed release of neurotransmitters and that these effects may be mediated by elevated intracellular calcium levels (Kater et al., 1988; Lipton and Kater, 1989; Fields et al., 1990b; Kater and Mills, 1991). The present study was undertaken to test if increases in intracellular calcium concentration or electrical activity are generally employed by neurons to stop or regulate neurite extension and growth cone morphology.

Electrical activity reversibly arrests neurite outgrowth and rapidly changes growth cone morphology in mouse dorsal root ganglion (DRG) and *Helisoma* buccal ganglion neurons (Cohan and Kater, 1986; Fields et al., 1990a). In related experiments, *Helisoma* and DRG neurons have been shown to stop growing in media that mimic some of the effects of electrical stimulation. Growth in chronically depolarizing conditions decreases DRG neurite outgrowth (Robson and Burgoyne, 1989); also, the application of 5-HT, which causes cells to fire action potentials, arrests neurite outgrowth from *Helisoma* B19 neurons (Haydon et al., 1987). Similarly, evidence has accumulated in recent years that growth in depolarizing media or neurotransmitter application affects neurite outgrowth from many cell types (for examples, see Anglister et al., 1982; Sussdorf and Campenot, 1986; Lankford et al., 1987; Pearce et al., 1987; Lipton et al., 1988; Mattson et al., 1988).

It has been suggested that electrical activity and neurotransmitter application regulate neurite outgrowth by increasing the calcium concentration in the growth cone ($[Ca]_{gc}$). Calcium has been shown to rise preferentially in growth cones in response to electrical activity (Cohan et al., 1987). Cohan et al. (1987) have suggested that electrical stimulation increases $[Ca]_{gc}$, which in turn stops outgrowth. In support of this hypothesis, both increases and decreases in $[Ca]_{gc}$ have been correlated with a reduction in neurite growth rate from some neurons. For example, applying the neurotransmitters 5-HT to *Helisoma* neurons (Murrain et al., 1990) or glutamate to cerebellar granule cells (Pearce et al., 1987) increases the intracellular calcium concentration ($[Ca]_i$) and inhibits outgrowth. In contrast, voltage-dependent calcium influx seems to be a signal for neurite extension from cultured retinal neurons (Suarez-Isla et al., 1984).

Are elevated activity rates and/or $[Ca]_{gc}$ universal cues for cells to stop growing? Several experimental results suggest that superior cervical ganglion (SCG) neurons might provide a counterexample to the general rule that outgrowth can be regulated by electrical stimulation or changes in the intracellular calcium concentration. First, Campenot and Draker (1989) have shown that extracellular calcium is not required at the growth cone for SCG neurite mobility. Second, Purves et al. (1986) have shown that in young adult mice SCG neurons continue to modify their dendritic arbor while they are electrically active. To test if high rates of electrical activity and elevated $[Ca]_{gc}$ stop outgrowth from SCG neurons, we stimulated individual SCG neurons at 10 Hz and measured both their intracellular calcium concentrations and their rate of outgrowth.

Materials and Methods

Cell culture. Rat pups (0–3 d old) were anesthetized with halothane and decapitated. The SCG were removed and transferred to Hank's balanced

Received Jan. 22, 1991; revised Aug. 2, 1991; accepted Aug. 7, 1991.

We thank Henry Lester, Jerome Pine, and David Tank for making their laboratories available for these studies and for supporting this research. We also thank David Anderson, Dennis Bray, Chi-Bin Chien, John Gilbert, Paul Patterson, and Jerome Pine for useful discussions and help with the manuscript.

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salts solution (HBSS) with penicillin/streptomycin and no divalent cations. After cleaning, the ganglia were incubated at 37°C in HBSS with 0.125% trypsin for 30 min in a 5% CO_2 environment. Following the incubation, the trypsin was inactivated by soaking the ganglia in modified Leibovitz's L-15 medium (L-15) with 32.5% horse serum for 10 min. The ganglia were transferred to L-15, shredded, triturated, and plated on polylysine-laminin–coated dishes at 3000 neurons/dish. Cells were allowed to settle for 30 min to 3 hr before experiments began.

Media. The modified L-15 was made by supplementing L-15 as described in Mains and Patterson (1973), except for the omission of bovine serum albumin and Methocel and the substitutions of 10% horse serum for rat serum and 4 ng/ml 2.5S NGF (Boehringer Mannheim) for 0.5 μg/ml 7S NGF. The medium used during all outgrowth experiments was $L\text{-}15_{air}$, which was prepared as L-15 except that sodium bicarbonate and serum were omitted, the pH was adjusted to 7.2 with NaOH, and the NGF concentration was reduced to 2 ng/ml. For experiments in high-K^+ L-15, NaCL was decreased from 137 to 92 mM and KCl was increased from 5.6 to 50 mM. To make high-K^+/high-Ca^{2+} L-15, the $CaCl_2$ concentration was also increased from 1.26 to 5 mM without a compensating adjustment in the concentration of other divalent cations. For experiments with the calcium ionophore bromo-A23187, the appropriate amount of a 20 μM stock solution (minimum dilution was 1:50) of the ionophore in dimethyl sulfoxide (DMSO) was added to the perfusing medium.

Substrate. Cells were grown on glass coverslips that were glued under a 6 mm hole in the bottom of 35 mm Petri dishes. The dishes were soaked for 30 min in fuming nitric acid, rinsed, and soaked overnight in Dulbecco's phosphate-buffered saline (DPBS) with 100 μg/ml polylysine (Sigma, P8905). Dishes were then rinsed, dried, and stored at 4°C until use. Just before plating, they were soaked in a 20 μg/ml solution of laminin (Sigma, L8263) in DPBS for 45 min and rinsed three times with DPBS.

Stimulation. Cells were stimulated extracellularly with patch pipettes (KG-33 glass, Garner Glass Co.). Tip diameters were about 1 μm, and electrode impedances were 5–15 MΩ. Pipettes were filled with high-potassium, low-sodium saline using a recipe modified from Marty and Neher (1983): 140 mM KCl, 2 mM $MgCl_2$, 11 mM EGTA-KOH (pH 7.3), 1 mM $CaCl_2$, and 10 mM HEPES, adjusted to pH 7.2 with KOH and to 320 mOsm with sucrose. Electrodes were positioned while monitoring the voltage drop produced by a hyperpolarizing current pulse (50 pA, 2 Hz). The test pulses were stopped as soon as a seal had formed, and the cell was observed for 30 min without stimulation to check for damage caused by the pipette. Stimulation experiments were performed only on cells that were undamaged by patching (i.e., continued to grow) during this control period. Cells were stimulated reliably at 10 Hz with pulses of 200–500 pA lasting 10–50 msec.

Outgrowth experiments. Outgrowth experiments were performed on a microscope stage with dishes perfused with $L\text{-}15_{air}$ at 20 ml/hr. The medium was warmed to 35 ± 1°C as it entered the dish through a channel in a heated stainless steel ring. The temperature was continuously monitored by a thermister located at the bottom of the dish. Under these experimental conditions, we found that the osmolarity of the medium remained constant.

For stimulation experiments, outgrowth was recorded by time-lapse 35 mm photography through a 20× phase-contrast objective. In experiments where the intracellular calcium concentrations were monitored, outgrowth was measured from fluorescence images recorded by a CCD camera (model 220 Photometrics) and stored on a computer (Macintosh IIx) (Regehr et al., 1989a). The extent of outgrowth was determined by measuring changes in growth cone position between successive images. Growth cone position was defined as the outermost point of the semicircle circumscribing the anterograde edge of the lamellapodia. Neurite lengths were measured to +2 μm.

Average growth rates were determined for a cell by measuring the growth rates of all neurites that could be observed and dividing by the number of neurites. Occasionally, a neuron was left with a stub that was observed immediately after plating and never grew or developed a growth cone during the course of an experiment. These stubs were likely remnants of processes incompletely sheared off during plating. Since they did not grow, they were not included in our calculation of average growth rate and did not contribute to the total outgrowth. Cells in which at least half of their processes could be observed continuously for 2 hr were used as controls.

Calcium measurements. Calcium measurements were performed using the acetomethoxy (AM) form of the calcium-sensitive fluorophore fura-2. Direct injections with the acid form could not be used because intracellular penetrations of SCG neurons stopped outgrowth. Neurons were stained by incubation in 1 ml of a 2 μM solution of fura-2 AM in $L\text{-}15_{air}$ at 37°C for 30 min. The 2 μM solution was made by adding 2 μl of a 1 mM fura-2 AM (Calbiochem) stock solution to 1 ml of $L\text{-}15_{air}$ and vortexing the solution to insure uniform mixing. Any remaining undissolved fura-2 AM was removed by centrifuging the solution in a microfuge for 1 min at 1000 rpm. The fura-2 AM stock solution was made up in 25% pluronic acid/75% dry DMSO and stored at −20°C for up to 2 weeks before use. After 30 min the cells were washed three times with $L\text{-}15_{air}$ and left to deesterify for 45 min before use. Staining of subcellular compartments became noticeable after about 3 hr and pronounced after about 5 hr. Therefore, experiments were performed within 3 hr of staining.

To minimize potentially cytotoxic effects of prolonged exposure to UV light, we limited the illumination times during experiments where both $[Ca]_i$ and outgrowth were measured. Typically we took no more than one 2 sec image every 5 min. Calcium measurements at either the growth cone or soma were averaged over a 3.3 μm^2 area. Calcium was measured in the soma at regions away from the nucleus, the site of pipette attachment, and the cell border.

Calcium levels were computed by the ratio method (Grynkiewicz et al., 1985), using R_{min} and R_{max} as determined in solution (Regehr et al., 1989a), and a dissociation constant of 225 nM. R_{min} and R_{max} differed from ratios determined using cells made permeable with ionophores. When 5 μM bromo-A23187 was used to permeabilize the cells, $R_{min} = 0.306 \pm 0.029$ and $R_{max} = 2.00 \pm 0.36$. When 200 nM ionomycin was used to permeabilize the cells, $R_{min} = 0.278 \pm 0.026$ and $R_{max} = 3.34 \pm 0.24$ (R_{min} and R_{max} were determined in solutions containing 1 nM and 5 mM Ca^{2+}, respectively). The larger R_{min}:R_{max} ratio obtained using ionomycin rather than bromo-A23187 to permeabilize the cells indicates that ionomycin was more effective at equilibrating the calcium concentration between the interior and exterior of the cell. It is not known whether differences between *in situ* and *in vitro* R_{max} values represent an inability of the ionophore to equilibrate Ca^{2+} across the membrane, or whether the differences represent a real difference in the response of fura-2 in the cell versus in saline. Deesterification was assessed to be essentially complete after 45 min, based upon the large R_{min}:R_{max} ratio obtained at that time (R_{min}:$R_{max} > 11$). For consistency with other papers in the field, no viscosity correction was used (Connor, 1986; Cohan et al., 1987).

Results

Neurites of cultured neonatal rat SCG neurons are formed by the steady advance of growth cones, which divide, apparently at random, to form branches (Bray, 1973). On polylysine–laminin substrates, processes were usually evident 30–90 min after plating. Initially, processes had large, flat growth cones that became more compact with time.

Stimulation

It was essential to have a reliable stimulation method to study the effects of activity on neurite outgrowth. In preliminary experiments, we found that intracellular and whole-cell electrode techniques stopped neurite outgrowth, and so we used extracellular patch pipettes to stimulate neurons noninvasively. To depolarize the cell without breaking down the membrane, a high-potassium, low-sodium saline was used in the pipette. This solution appeared to depolarize the portion of the membrane inside the pipette, opening noninactivating channels, because the pipette potential approached the cell's resting potential, −70 mV relative to the bath, after a seal was obtained. The open channels reduced the impedance of the patch and allowed enough current to pass to stimulate the cell without damaging the membrane (see Regehr et al., 1989b, for a detailed explanation). The reduced patch impedance also increased the signal-to-noise ratio for recording evoked action potentials. SCG neurons did not spontaneously fire action potentials. Cells were rejected if we saw damage upon patch formation or were unable to evoke

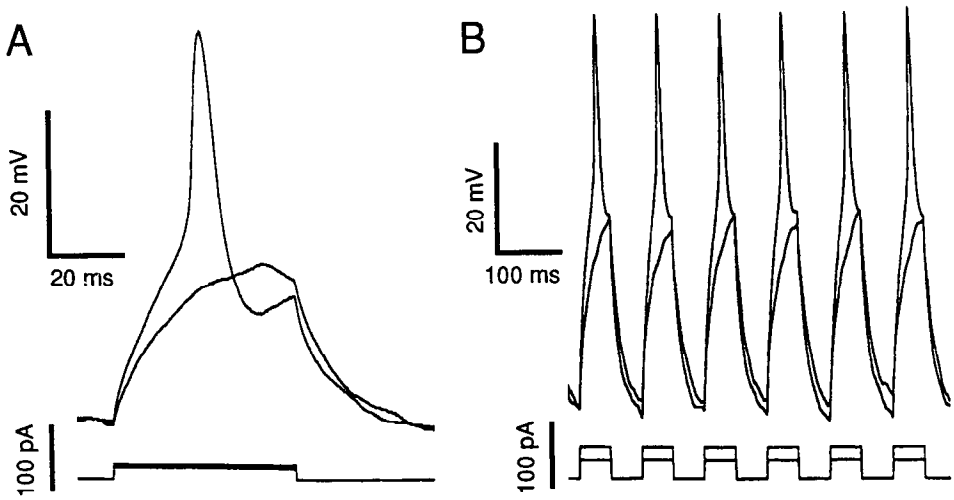

Figure 1. SCG neurons can be reliably stimulated to fire action potentials using a membrane-depolarizing cell-attached patch. *a,* The *top* voltage traces display the potentials recorded when the stimulating current was set just above and just below threshold with 1 Hz stimulation. The current traces are shown at the *bottom. b,* Reliable stimulation was also obtained at 10 Hz, as shown in sub- and suprathreshold voltage and current traces.

action potentials without breaking down the membrane.

Typically, action potential amplitudes recorded with extracellular pipettes were between 4 and 40 mV in magnitude. Figure 1 shows recordings from a cell stimulated at 1 and 10 Hz: the bottom trace is with stimulation just below threshold, and the top trace is with the stimulus current increased to just above threshold. The magnitude and shape of the action potentials varied over the course of an experiment, presumably as the impedance of the seal and patch fluctuated. All experiments were performed using 10 Hz chronic stimulation because preliminary experiments had shown that stimulation at 1 and 2 Hz did not affect neurite outgrowth rates and 10 Hz was the fastest rate at which we could consistently stimulate the neurons.

Outgrowth during stimulation

To determine if electrical activity is likely to regulate the rate of process outgrowth from SCG neurons, we monitored outgrowth before, during, and after stimulation (Fig. 2). Throughout these experiments, both experimental and control neurons continued to grow and branch. Figure 3 is a plot of total outgrowth from all neurites from the cell in Figure 2: total outgrowth is the sum of the measured outgrowth from all neurites during all time intervals. For comparison, the total outgrowth from a control cell over the same time period is also given (original images are not shown). Cumulative growth was very similar between the two cells and showed a slight increase in slope with time due to neurite branching.

In calculating the average growth cone outgrowth rate (average growth rate), all processes were included, whether they grew steadily or retracted (16 of 251 processes retracted, only one reversibly). The average growth rate for all cells declined with time. For the stimulated or experimental cells, average growth rates during successive 30 min periods were 34 ± 2 [62 growth cones (gc) on 7 neurons (n), ± SEM], 28 ± 2 (71gc on 7n), 25 ± 2 (73gc on 7n), and 20 ± 2 μm/hr (46gc on 6n). These time periods correspond to, respectively, growth without a pipette or stimulation, with a quiescent pipette, with stimulation at 10 Hz, and after the cessation of stimulation. Average growth rates of control cells showed the same decline with time as experimental cells (see Fig. 4). Average growth rates from control cells during successive 30 min time periods were 24 ± 3 (42gc on 9n), 21 ± 2 (40gc on 9n), 18 ± 3 (36gc on 9n), and 16 ± 2 μm/hr (32gc on 9n). A comparison of the average growth rates of control and experimental cells is given in Figure 4. The decrease in the average growth rate was paralleled by a decrease in the size of the growth cones.

Intracellular calcium measurements and concentrations

Before determining how electrical stimulation affected $[Ca]_i$, we determined the calcium levels for control cells. $[Ca]_{soma}$ was 96 ± 4 nM and $[Ca]_{gc}$ was 127 ± 33 nM (21gc from 5 cells). These levels were similar to those reported for frog SCG (Lipscombe et al., 1988), rat diencephalon (Connor, 1986), and *Helisoma* neurons (Cohan et al., 1987). Calcium levels varied across the soma, with the region corresponding to the nucleus having a slightly lower calcium level (e.g., see Fig. 6*A*).

We estimate that 25% of our staining was membrane associated or compartmentalized, based on comparisons of fluorescence before and after obtaining whole-cell recordings with a saline-filled pipette. All of the fura-2 should rapidly diffuse into the essentially infinite volume of the patch pipette, leaving the cell nonfluorescent; this did not occur. In three cells, roughly 25% of the fluorescence remained intracellular after a 15 min whole-cell recording.

Growth cone calcium and neurite outgrowth

To determine the effect of electrical stimulation on $[Ca]_{gc}$, we measured the response of $[Ca]_{gc}$ to 10 Hz stimulation (Figs. 5, 6). In the absence of electrical stimulation, patch electrode placement did not affect $[Ca]_{gc}$. Occasionally a small increase of less than 40 nM in $[Ca]_{soma}$ was observed localized to the area adjacent to the pipette. Chronic electrical stimulation at 10 Hz increased $[Ca]_{gc}$ first, with $[Ca]_{soma}$ following slowly as shown in two different cells in Figures 5 and 6*A–E*. Growth cone calcium increased from 128 ± 23 nM to peak levels of 575 ± 165 nM in 10–15 sec (±SD; 16gc on 4n). Within 2–3 min, $[Ca]_{gc}$ decreased to steady state levels of 336 ± 61 nM. $[Ca]_{soma}$ increased more slowly from prestimulus levels of 65 ± 14 nM to peak values of 364 ± 77 nM, with a plateau level of 332 ± 93 nM. When stimulation ended, calcium returned to prestimulus levels within minutes (Figs. 5, 6*F*).

We also determined the calcium levels reached during extended periods of stimulation (see Fig. 5 for an example with one cell). Since we could not reduce the illumination intensity

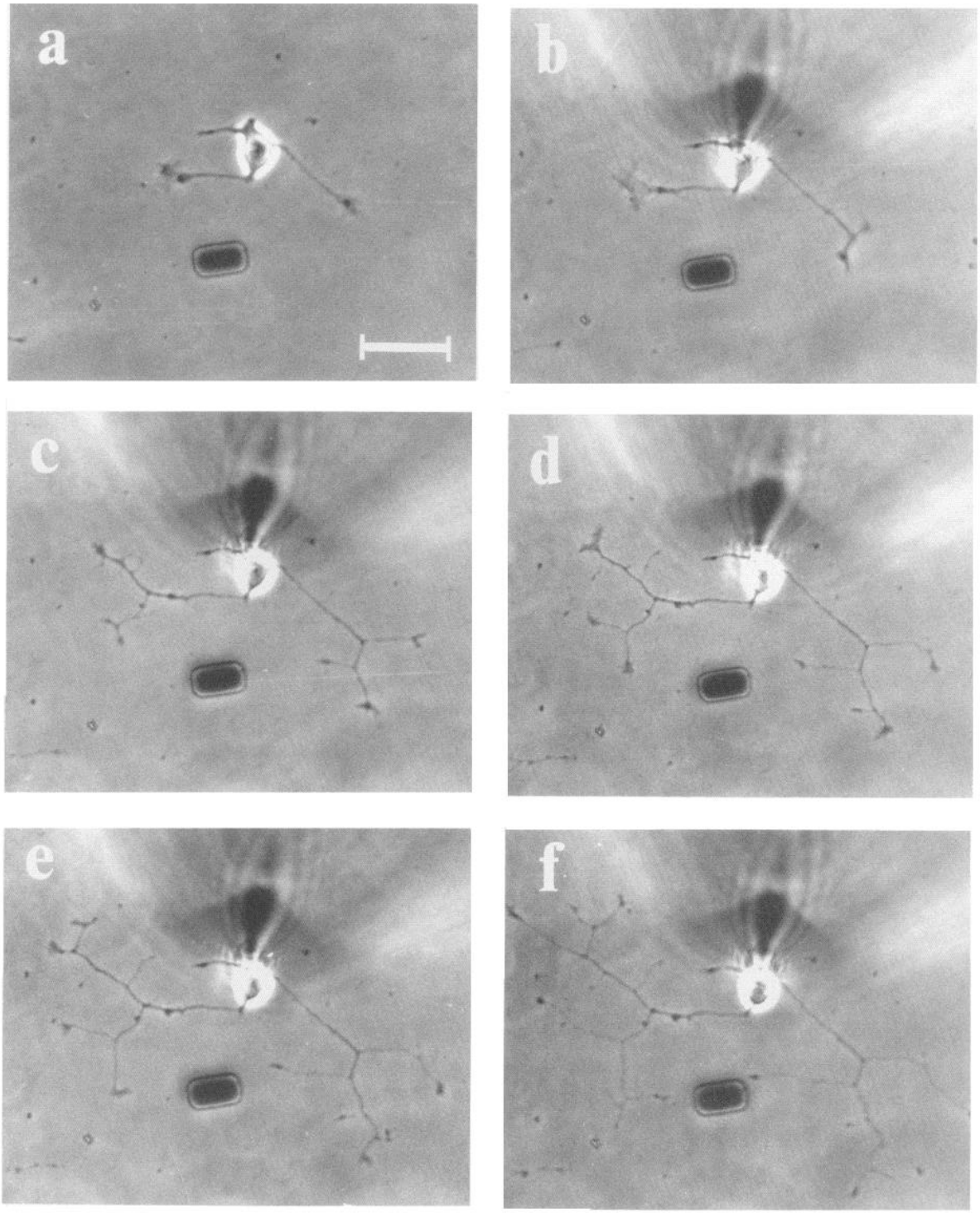

Figure 2. SCG neurons continue to grow during 10 Hz electrical stimulation. Photographs *a–f* were taken every 30 min. They began at $t = 0$ min (*a*). A gigaohm seal was formed between the neuron and the pipette at $t = 30$ min (*b*); 10 Hz stimulation began at $t = 60$ min (*c*), continued at $t = 90$ min (*d*), and stopped at $t = 120$ min (*e*). Growth 30 min after the cessation of stimulation is shown in *f*. All processes continued to grow and branch throughout the experiment. The *dot* is part of gold grid pattern used for alignment. Scale bar, 50 μm.

further and retain the necessary signal-to-noise ratio, we conducted another series of experiments during which images were taken every 10 min to prevent a reduction in growth rate. $[Ca]_{gc}$ increased from 109 ± 44 nM to steady state values of 294 ± 101 nM, and $[Ca]_{soma}$ increased from 75 ± 51 to 283 ± 160 nM (±SD; 24gc on 8n). Steady state values were determined from 10 to 40 min after the beginning of stimulation. The SCG neurons continue growing during these experiments as shown in Figure 7.

We attempted to determine the effect of increasing the $[Ca]_{gc}$

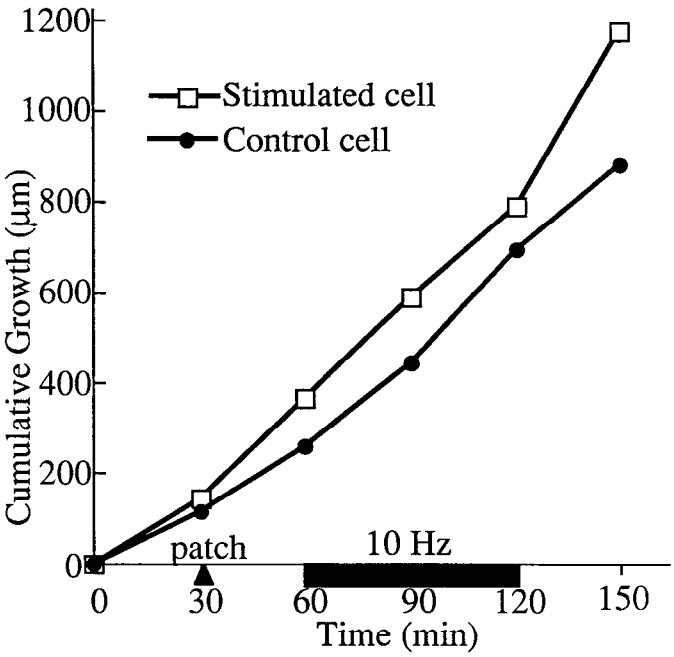

Figure 3. Cumulative growth is unaffected by 10 Hz electrical stimulation. The cumulative growth (summed increase in the length of all processes) for the cell shown in Figure 2 and an unstimulated cell (not shown) is plotted against time. The cumulative growth rate increases with time due to the addition of new branches.

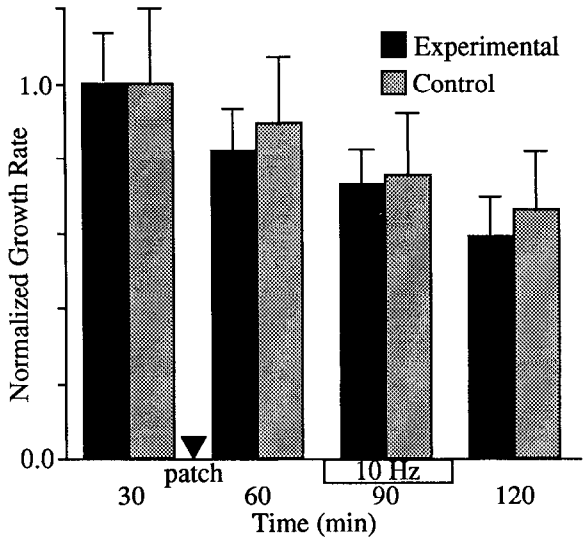

Figure 4. Average neurite growth rate is unaffected by stimulation at 10 Hz. The normalized average growth rates for experimental cells are plotted as a function of experimental condition: without pipette, with pipette, stimulated, and after stimulation. Each condition was maintained for 30 min. For comparison, average growth rates over successive 30 min intervals for control cells are also plotted. To facilitate comparison, the average growth rate for each growth cone was normalized to its initial rate of growth as measured during the control period. *Error bars* represent the SEM of the average normalized growth rate. See text for the absolute growth rates and number of neurons or growth cones in each condition.

above the concentration attainable with tonic stimulation by growing SCG neurons in depolarizing media or with calcium ionophores. When the solution was abruptly switched from standard medium to high-K^+ saline or high-K^+/high-Ca^{2+} saline, the $[Ca]_{gc}$ briefly rose to greater than 300 nM, but within minutes it returned to steady state levels of less than 200 nM. Unable to increase the $[Ca]_{gc}$ to levels above 300 nM with depolarizing medium, we attempted to raise the $[Ca]_{gc}$ further by growing the cells with the calcium ionophore bromo-A23187. In the presence of 20–100 nM bromo-A23187 (1.26 mM Ca^{2+} in the medium), the $[Ca]_{gc}$ increased to less than 200 nM. Increasing the concentration of bromo-A23187 to 400 nM produced large calcium accumulations that oscillated between about 200 and 500 nM. Therefore, we were unable to measure the effect of steady state calcium concentrations above that attained with tonic stimulation by growing SCG neurons in these conditions.

Discussion

Our results suggest that neither electrical activity nor sustained $[Ca]_{gc}$ of about 300 nM affect neurite outgrowth rates from SCG neurons. Electrical stimulation at the fastest feasible rate, 10 Hz, produced no observable changes in outgrowth from SCG neurons but did cause an increase in $[Ca]_{gc}$. Even when steady state calcium levels increased by a factor of 3 from resting levels of 128 ± 23 nM to steady state levels of 336 ± 61 nM, outgrowth was unaffected.

Electrical stimulation

The response of SCG neurons to stimulation is strikingly different from that of the other cell types studied, mouse DRG neurons and *Helisoma* buccal ganglion neurons. DRG neurons immediately retract their filopodia and lamellipodia when electrically stimulated at 2.5–10 Hz (Fields et al., 1990a). For example, in response to 10 Hz stimulation, DRG neurons retract their filopodia and lamellipodia and 75% of their neurites. B19 cells from the buccal ganglion of *Helisoma* also stop neurite outgrowth (but do not retract) when stimulated with a cell-attached patch pipette at 4 Hz (Cohan and Kater, 1986).

$[Ca]_{gc}$ and outgrowth

Since activity-induced growth inhibition has been correlated with changes in $[Ca]_{gc}$ (Cohan et al., 1987) and it has been suggested that increases in $[Ca]_{gc}$ cause the observed inhibition of outgrowth, we determined whether activity increases the $[Ca]_{gc}$ in SCG neurons. In response to 10 Hz chronic stimulation, SCG neurons increase their $[Ca]_{gc}$ transiently from 128 ± 23 to 575 ± 165 nM before plateauing at 336 ± 61 nM. These changes are similar to those that correlated with an inhibition of outgrowth from *Helisoma* neurons. $[Ca]_{gc}$ rises transiently in *Helisoma* B5 neurons from about 100 to 300–400 nM before plateauing at 225–325 nM with 3 Hz stimulation (Cohan et al., 1987), while 4 Hz stimulation stops outgrowth from these same cells (Cohan and Kater, 1986). Similarly, applying 5-HT to *Helisoma* B19 neurons, at concentrations known to stop outgrowth (Haydon et al., 1987), causes calcium to rise from 125 to 330 nM (Cohan et al., 1987). Transient increases in $[Ca]_{gc}$ of four- to sevenfold and steady state changes of three- to fivefold occur in *Helisoma* buccal ganglion (Cohan et al., 1987), DRG (Field et al., 1990b), and SCG neurons, but only are correlated with an inhibition of outgrowth in the first two cell types.

Why do SCG neurons respond differently?

Electrical stimulation does not stop outgrowth from SCG neurons as it does from DRG and *Helisoma* neurons. Since electrical stimulation causes similar increases in $[Ca]_{gc}$ in SCG, DRG, and *Helisoma* neurons, it is unlikely that the $[Ca]_{gc}$ alone is respon-

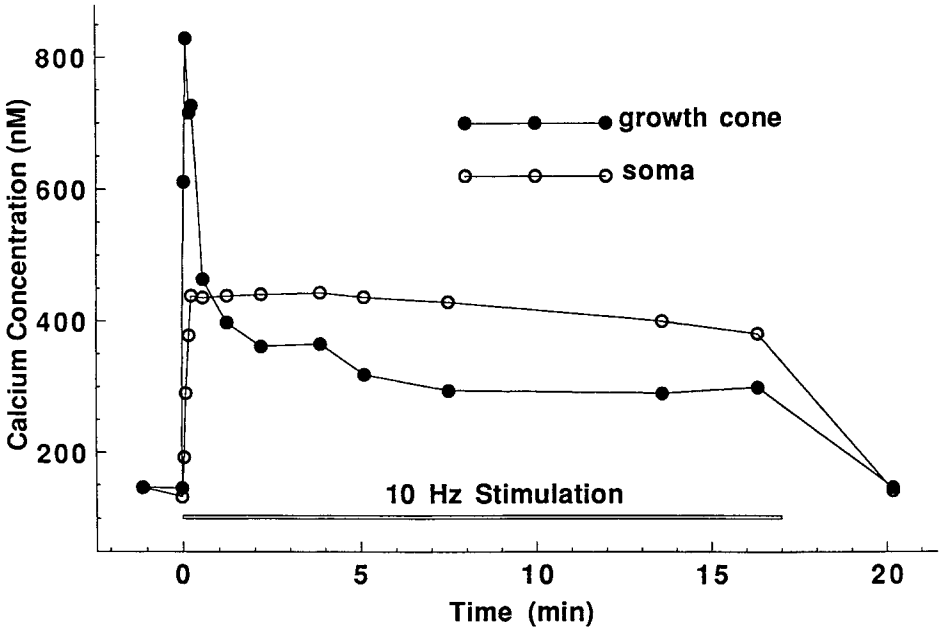

Figure 5. $[Ca]_{gc}$ and $[Ca]_{soma}$ as a function of time during 10 Hz electrical stimulation for an SCG neuron (as indicated by the *open bar* beneath the plot). The calcium concentrations almost return to prestimulus level just 3 min after the cessation of stimulation.

sible for these differences. One possible explanation is that the calcium sensitivity of outgrowth from SCG neurons may greatly differ from the sensitivity of outgrowth from DRG or *Helisoma* neurons, and 10 Hz electrical stimulation did not increase $[Ca]_{gc}$ enough to stop outgrowth from SCG neurons. It is also possible that SCG and other neurons may employ cues other than intracellular calcium to control outgrowth. For example, there is evidence that bag cell neurons from *Aplysia* use cAMP to regulate growth cone structure (Forscher et al., 1987).

A reinterpretation of the observed calcium increases may also explain apparent differences in the calcium sensitivity of outgrowth between SCG and DRG or *Helisoma* neurons. Perhaps it is high local concentrations of calcium, $[Ca]_i$, rather than bulk calcium levels that stop outgrowth in DRG and *Helisoma* neurons. Many cellular processes that have a low calcium affinity are activated by high calcium concentrations. Release of neurotransmitter is an example of such a process: it is only near the mouth of open voltage-dependent calcium channels that calcium reaches sufficient levels to support significant release of neurotransmitter, whereas the average calcium in a presynaptic terminal is sufficient to support only extremely low levels of release (Fogelson and Zucker, 1985; Simon and Llinas, 1985). Variations in $[Ca]_i$ such as elevations near calcium channels or clusters of channels (Lipscombe et al., 1988; Silver et al., 1990) would be expected to exist even in a relatively thin structure, such as a growth cone. Fura-2 imaging is relatively insensitive to $[Ca]_i$ due in part to spatial and temporal averaging. No experiments have yet been performed that would clearly distinguish between bulk calcium levels and high $[Ca]_i$ being responsible for stopping outgrowth. We, however, interpret recent experiments demonstrating the involvement of calmodulin (Polak et al., 1991) in the activity-dependent stoppage of outgrowth to suggest that high $[Ca]_i$ may be involved. Calmodulin is a protein with multiple calcium-binding sites and relatively low calcium affinity (Manalan and Klee, 1984). It is thought to be activated by calcium in a nonlinear manner, so that high $[Ca]_i$, greater than those measured in the bulk cytoplasm, may be needed to activate this protein. If proteins such as calmodulin regulate outgrowth, differences either in the localization of calcium ions or in the calcium affinity of such calcium-activated proteins could explain the observed differences in the regulation of outgrowth between cell types.

How do these in vitro *results relate to growth* in vivo*?*

It is unlikely that elevated rates of electrical activity regulate neurite elongation from SCG neurons *in vivo,* since SCG activity rates are likely to be substantially lower than the 10 Hz that failed to inhibit outgrowth *in vitro.* The estimate of *in vivo* activity rate is based on observed spontaneous activity rates of sympathetic preganglionic neurons in both frog and cat (Iggo and Vogt, 1960; Blackman et al., 1963; Polosa, 1968; Janig and Schmidt, 1970; we were unable to find the corresponding data from unanesthetized rat SCG neurons). The supposition that electrical activity does not regulate neurite outgrowth from SCG neurons *in vivo* is consistent with the observation that the neurons continue to modify their dendritic arbors throughout young adulthood, when they are electrically active (Purves et al., 1986). Based on *in vitro* experiments, it is possible that activity regulates outgrowth *in vivo* from *Helisoma* and DRG neurons. Whether these neurons actually employ this mechanism in development has not been established.

The independence of neurite outgrowth from electrical activity accompanied by increases in calcium may be a feature common to many CNS neurons. For example, mitral cells of the olfactory bulb are similar to SCG neurons in that their dendritic arbors continue to undergo modification in young adults (Pomeroy et al., 1990). In addition, retinal ganglion cells extend toward the tectum and form functional synapses even with the elimination of TTX-sensitive action potentials (Harris, 1980), although the segregation of the projections in the tectum may be activity dependent (Schmidt and Edwards, 1983; Reh and Constantine-Paton, 1985). These studies imply that it may be simplistic to assume that even if electrical activity or the accompanying rise in calcium regulates some portion of neuronal development, it is necessarily going to regulate neurite elongation.

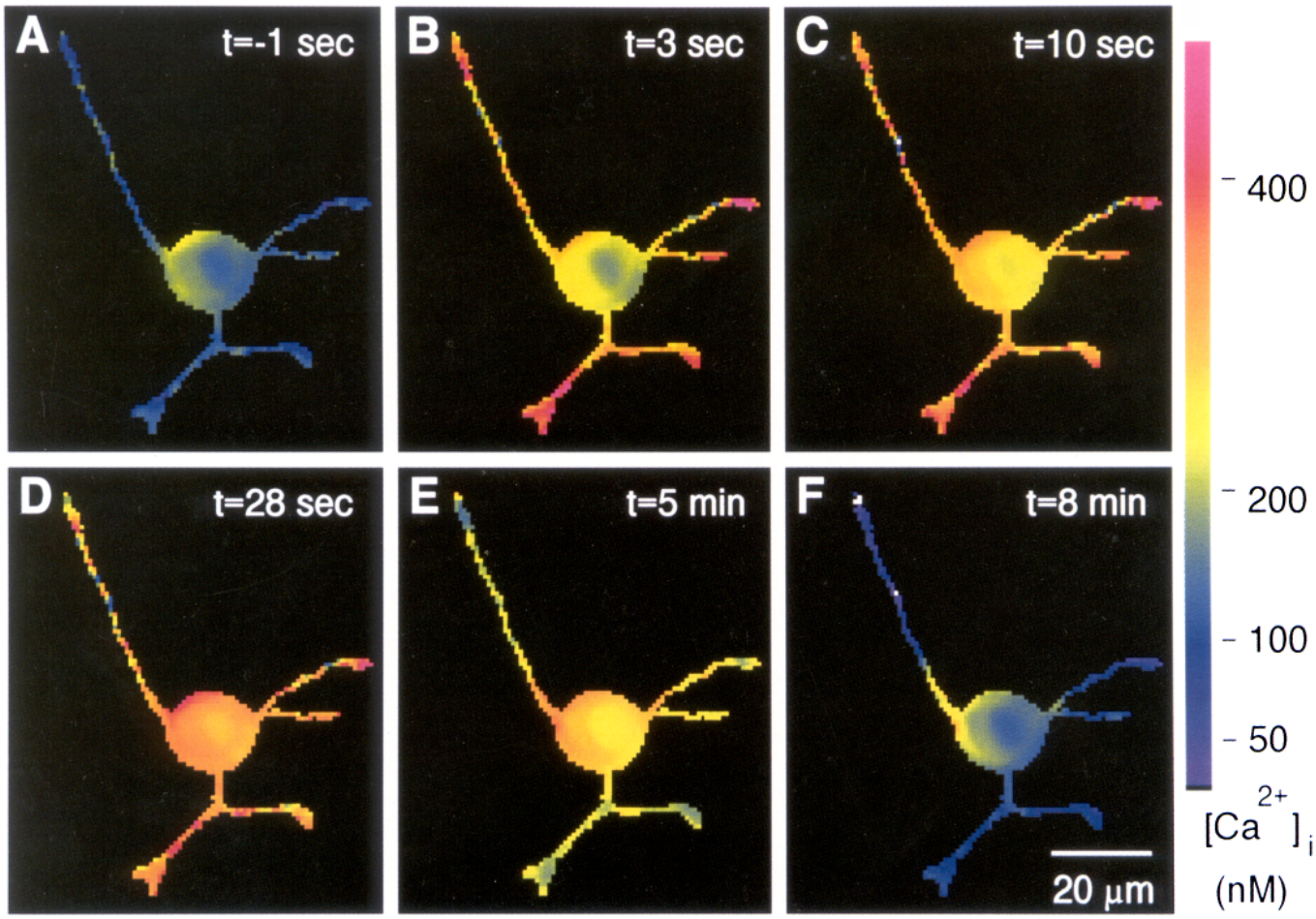

Figure 6. Electrical stimulation at 10 Hz increases the intracellular calcium concentration as measured using fura-2 AM. Calcium levels are represented in pseudocolor images (the *bar to the right* correlates calcium concentration with color). Stimulation began at $t = 0$ sec and continued until $t = 5$ min. As shown in *B* and *C*, intracellular calcium levels rise in the growth cone before rising in the soma. Because the pipette did not affect the $[Ca]_{soma}$, the site of pipette attachment is not visible in the photographs.

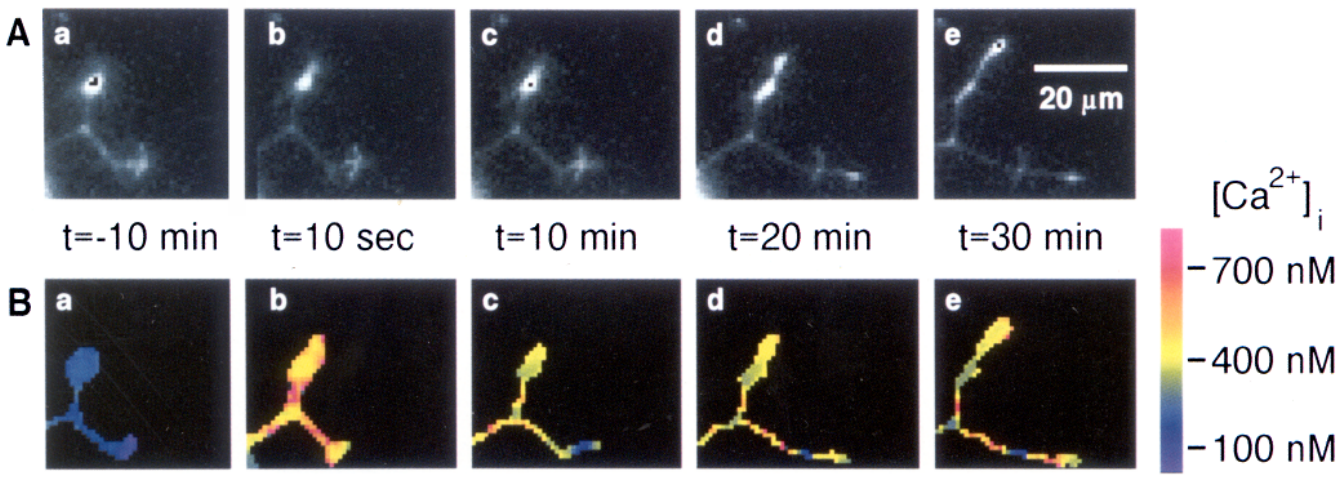

Figure 7. Neurite outgrowth continues during electrical stimulation at 10 Hz even when $[Ca]_{gc}$ remains elevated at over 400 nM. Series *A* shows successive fluorescent images of the two growth cones; series *B* shows pseudocolor calcium images. The soma is not in the field of view.

Conclusions

The observation that SCG neurites continue to elongate during stimulation suggests that increased rates of electrical activity and increases in $[Ca]_{gc}$ are not universal signals to stop neurite elongation. Clearly, the regulation of outgrowth *in vivo* is an involved task. It seems reasonable that a variety of mechanisms will be used to regulate it; only one of these mechanisms is electrical activity accompanied by an increase in $[Ca]_{gc}$.

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