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Electrically and Chemically Mediated Increases in Intracellular Calcium in Neuronal Growth Cones

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In the present report we used the calcium indicator fura-2 to compare intracellular levels of free calcium in growth cones of isolated *Helisoma* neurons under a variety of experimental conditions. We tested whether 2 different signals that inhibit growth cone motility—action potentials and serotonin—changed calcium levels in growth cones. Electrical stimulation of the cell body caused a rise in calcium levels at the growth cone. After brief stimulation, calcium levels quickly recovered to normal values, whereas longer stimulation periods required longer recovery times. The application of serotonin to growth cones caused an increase in calcium levels that was selective for growth cones of neurons whose outgrowth was inhibited by serotonin, but not for neurons whose outgrowth was not affected. We also found that motile growth cones had higher free calcium levels than growth cones that had spontaneously stopped growing. Furthermore, the distribution of calcium in neurons that contained motile growth cones was heterogeneous; calcium levels were always higher in the growth cone than in the neurite or soma. These data indicate that calcium levels in growth cones vary in different states of outgrowth and that calcium levels can be modulated by both electrical and chemical signals.

Calcium has long been regarded as a plausible candidate in the control of growth cone motility (Bray, 1973; Llinás, 1979). Gunderson and Barrett (1980) provided evidence that the turning response to nerve growth factor required elevated calcium levels in the growth cone. More recent evidence, using direct monitoring of calcium levels in cultured mammalian neurons, has shown that calcium is elevated in neurons that are actively extending neurites (Connor, 1986). Moreover, calcium concentration has tended to be higher at the growth cone than at the soma or proximal neurites of growing neurons. These studies are supported by other investigations, which have indicated the presence of voltage-sensitive ion channels in the growth cone membrane (Grinvald and Farber, 1981; Anglister et al., 1982; Bolsover and Spector, 1986). Together, these reports show that calcium can enter the growth cone and that motile responses are correlated with changes in calcium levels.

A further test of whether calcium is involved in growth cone motility would require the ability to experimentally manipulate the motile behaviors of growth cones and consequently observe alterations in calcium levels. Recently, studies of isolated, identified neurons from the snail *Helisoma* have demonstrated that 2 signals prominent in adult nervous systems, neurotransmitters and action potentials, can convert growing neurons to a nongrowing state. Moreover, *Helisoma* neurons spontaneously stop growing 4–7 d after they are plated into culture (Cohan et al., 1985; Hadley et al., 1985). These changes from growing to nongrowing states offer the opportunity to study how motility may be regulated in growth cones. Our strategy in the present paper has been to ask whether signals that have been shown to inhibit the motility of growth cones of *Helisoma* neurons also cause a change in the concentration of free calcium at the growth cone.

One method of inhibiting growth cone motility in *Helisoma* neurons involves the use of the neurotransmitter serotonin. This neurotransmitter has neuron-specific effects on growth cone behaviors (Haydon et al., 1984). The motility of growth cones of identified buccal neuron B19 in cell culture is inhibited by the application of serotonin to the bathing medium. By contrast, application of serotonin has no effect on growth cones of buccal neuron B5. These observations have recently been extended to other neurotransmitters and a larger set of neurons (McCobb et al., 1985). The finding that focal application of serotonin to isolated growth cones of neuron B19 also suppresses their motility (Haydon et al., 1984) indicates the importance of local mechanisms at the growth cone in regulating motility.

A second method of inhibiting growth cone motility in *Helisoma* neurons is through electrical activity. The generation of action potentials has been demonstrated to abruptly and reversibly inhibit growth cone motility and neurite elongation in different identified neurons in *Helisoma* (Cohan and Kater, 1986). Inasmuch as both serotonin (McCobb and Kater, 1986) and action potentials evoke ionic currents in these neurons, the possibility exists that both serotonin and action potentials produce their effect by movement of ions across the growth cone membrane.

The availability of fluorescent calcium indicators that can be loaded into nerve cells has made the present study possible. We employed fura-2, a recently developed calcium indicator that is a powerful quantitative probe of free calcium changes in living cells (Grynkiewicz et al., 1985; Connor, 1986; Tsien, 1986). The ester form of this dye freely crosses cell membranes and, once in the cytoplasm, it is de-esterified and thus trapped internally. Alternatively, the ionic form of this dye can be injected directly

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into the cytoplasm. This dye, together with high resolution imaging technology, has made it possible to quantify variations in free calcium in different regions of the same neuron as well as in similar regions within different neurons.

Our present results confirm earlier observations (Connor, 1986) of increased levels of calcium in the growth cones of growing neurons, compared to those in nongrowing neurons. We extend these observations to growth cones that stopped growing as a result of the application of serotonin or the generation of action potentials. We demonstrate that action potentials in different identified *Helisoma* neurons cause large increases in free calcium levels in growth cones. Serotonin also causes large increases in calcium levels, but only in growth cones of those identified neurons whose outgrowth is known to be inhibited by serotonin. Our data suggest that growth cone motility is related to free calcium levels within the growth cone.

Materials and Methods

Experiments were performed on adult (13–17 mm shell diameter) specimens of the albino (red) form of the snail *Helisoma trivolvis,* which were reared in the laboratory and maintained as inbred stocks. Snails were housed in aquaria at room temperature (27°C) and fed trout chow and lettuce. They were deshelled and prepared for sterile dissection as outlined elsewhere (Wong et al., 1981). Dissections (Kater and Kaneko, 1972) were made aseptically and buccal ganglia were removed from the animals and prepared for neuronal isolation for cell culture.

Cell isolation and culture. Details of the culture procedure for isolating snail neurons are given in Haydon et al. (1984). Briefly, buccal ganglia were soaked in a 0.2% trypsin solution for a period of 30 min, followed by 15 min in a 0.2% solution of trypsin inhibitor. After enzymatic treatment the somata of single visually identifiable neurons were exposed by making a slit in the connective sheath (surrounding the ganglion) adjacent to the neuron somata with an electrolytically sharpened tungsten microknife. Cell bodies with attached axons were removed from ganglia by gentle suction with a fire-polished micropipette. Isolated neurons were transferred to preconditioned culture dishes (Falcon 3001) containing 2 ml of brain-conditioned medium (Wong et al., 1981), where they adhered to a polylysine-coated glass coverslip substratum. Preconditioned culture dishes were produced by incubating 2 ml of defined medium with 4–6 brains. At 72 hr the brains were removed and the dishes were refrigerated until plating.

Twelve hours after plating the neurons, the culture dishes were refrigerated (4°C) to delay neurite outgrowth. *Helisoma* neurons stored in this manner produce elongating neurites with growth cones within 12 hr of exposure to room temperature. Dishes were transported to Bell Labs (where calcium measurements were performed) in a cooled container, refrigerated upon arrival, and removed from the refrigerator immediately before use.

In some experiments serotonin was applied focally to specific regions of neurons with positive pressure (1–10 cm H_2O) from a pipette (tip diameter, 1–2 μm) that contained serotonin (10^{-4}–10^{-5} M) and was positioned 10–30 μm from the growth cone.

Electrophysiology. Microelectrodes for intracellular recording were made from fiber-filled, thin-walled glass (Haer) that was pulled on a Brown-Flaming micropipette puller. Microelectrodes were filled with 3 M KCl (20–40 MΩ DC resistance) and were connected to bridge-balanced preamplifiers. Recordings were displayed on an oscilloscope and permanently stored on a chart recorder (Gould Instruments) or digital oscilloscope (Nicolet Instruments). In the present study, care was taken to minimize the spontaneous generation of action potentials during impalement with microelectrodes by applying 1–2 nA of hyperpolarizing current.

Analysis of intracellular free calcium concentrations. The fluorescent calcium indicator, fura-2, was loaded into neurons by 1 of 2 methods: incubation in media containing fura-2/AM, the membrane-permeable form (Tsien, 1981; Tsien et al., 1982; Grynkiewicz et al., 1985), which was used for most of the experiments, or injection of the ionic form directly into neuronal somata with a patch pipette. For loading the membrane-permeable form (hereafter referred to as "/AM loading"), the plated cells were exposed for 1–1.5 hr to a suspension of fura-2/AM dissolved in dimethylsulfoxide (DMSO) and mixed with conditioned media at a nominal concentration of 2 μM fura-2 and 0.3% DMSO. The preparations were then rinsed with new conditioned media and allowed to stand for a minimum of 1.5 hr to allow de-esterification of the indicator. All incubations were at room temperature. Indicator amounts in the neurites were estimated by comparing their fluorescence when loaded with fura-2 to the fluorescence of fura-2 at a known concentration in thin channels etched into microscope coverslips (Connor, 1986). Loadings between 50 and 100 μM were obtained.

The ionic form was injected as follows: The tip of a patch pipette (tip diameter approximately 1 μm) was filled with fura-2 (400 μM) in *Helisoma* intracellular saline (Cohan and Kater, 1986). Fura-2 was the only calcium chelator in the electrode. A seal was obtained on the cell membrane and the membrane within the pipette was ruptured by suction and negative voltage pulses. In most cases it was necessary to apply large negative pulses intermittently to keep the pipette tip open. A negative current of 0.5–1 nA also appeared to speed the flux of indicator into the cell. Generally, it took 15–20 min to achieve levels comparable to the /AM loading technique. The patch electrode was then removed to avoid excessive dialysis of the cell interior. Intrinsic fluorescence of the cells was less than 3% of the indicator fluorescence (340–380 nm excitation).

Neurons were viewed with Nomarski optics or anaxial illumination, the latter method providing better images of growth cones. Individual growth cones of a neuron were continuously monitored under high magnification using a low-light video microscopy system, as overexposure can inhibit outgrowth (Kater et al., 1986). The imaging system and procedures have been described in more detail elsewhere (Connor, 1986). Its main components are a cooled, charge-coupled device (CCD) camera (Sequin and Thompsett, 1975; Tyson et al., 1982; Tyson and Boeshaar, 1983), an LSI 11-73-based computer, a Zeiss IM-35 microscope, and a Nikon UV-F 40× objective. Spatially resolved calcium measurements were made by acquiring 2 images of the same location, made at different excitation wavelengths, 340 and 380 nm, correcting for background and dark current, and then forming a ratio image from them (340:380). Data acquisition required 2–3 sec for a pair of images. Fluorescence was measured using a 480 nm long-pass filter. In principle, ratio measurements eliminate the effects of pathlength (thickness), excitation intensity, and concentration difference in the preparation, leaving a signal that is a measure of calcium concentration (cf. Grynkiewicz et al., 1985; Keith et al., 1985). In practice, these effects are reduced by factors >100 for light pathlengths of the order encountered in neurites (J. A. Connor, unpublished observations). The minimum block size used to compute statistics on the image was 10 × 10 pixels.

Indicator fluorescence ratios can be converted to calcium concentration using the following equation (see Grynkiewicz et al., 1985)

$$[Ca^{2+}] = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\frac{F_0}{F_s},$$

where K_d is the equilibrium dissociation constant for fura-2, the R terms are fluorescence ratios using 340 and 380 nm excitation (i.e., $R = F_{340}/F_{380}$), and F_0/F_s is the fluorescence ratio obtained at low and saturating calcium levels with 380 nm excitation. R_{max} for saturating levels of calcium was 11.7, R_{min} obtained for solutions with no added calcium and 5 mM EGTA was 0.5, and F_0/F_s was 7.5. The ionic strength and experimental temperature for *Helisoma* are approximately the same as for experiments on amphibians reported by Williams et al. (1985). This makes the appropriate *in vitro* K_d for fura-2 calcium binding approximately 214 nM. Owing to the very large size of the *Helisoma* neuron cell bodies, we have not had success with *in vivo* calibrations of the type reported by Williams et al. (1985), in which ionomycin was used to control cytoplasmic calcium levels of smooth muscle cells.

Results

A total of 40 neurons were examined in the present series of experiments: neurons B19 (n = 18), B4 (n = 4), B5 (n = 11), and unidentified neurons (n = 7) that were not specifically typed during the experimental procedure. Thirteen of the neurons were examined after they had been exposed to room temperature for periods exceeding 20 hr and had grown to a stable morphology. The remaining 27 neurons were examined within 12 hr of exposure to room temperature and had motile growth cones. Sev-

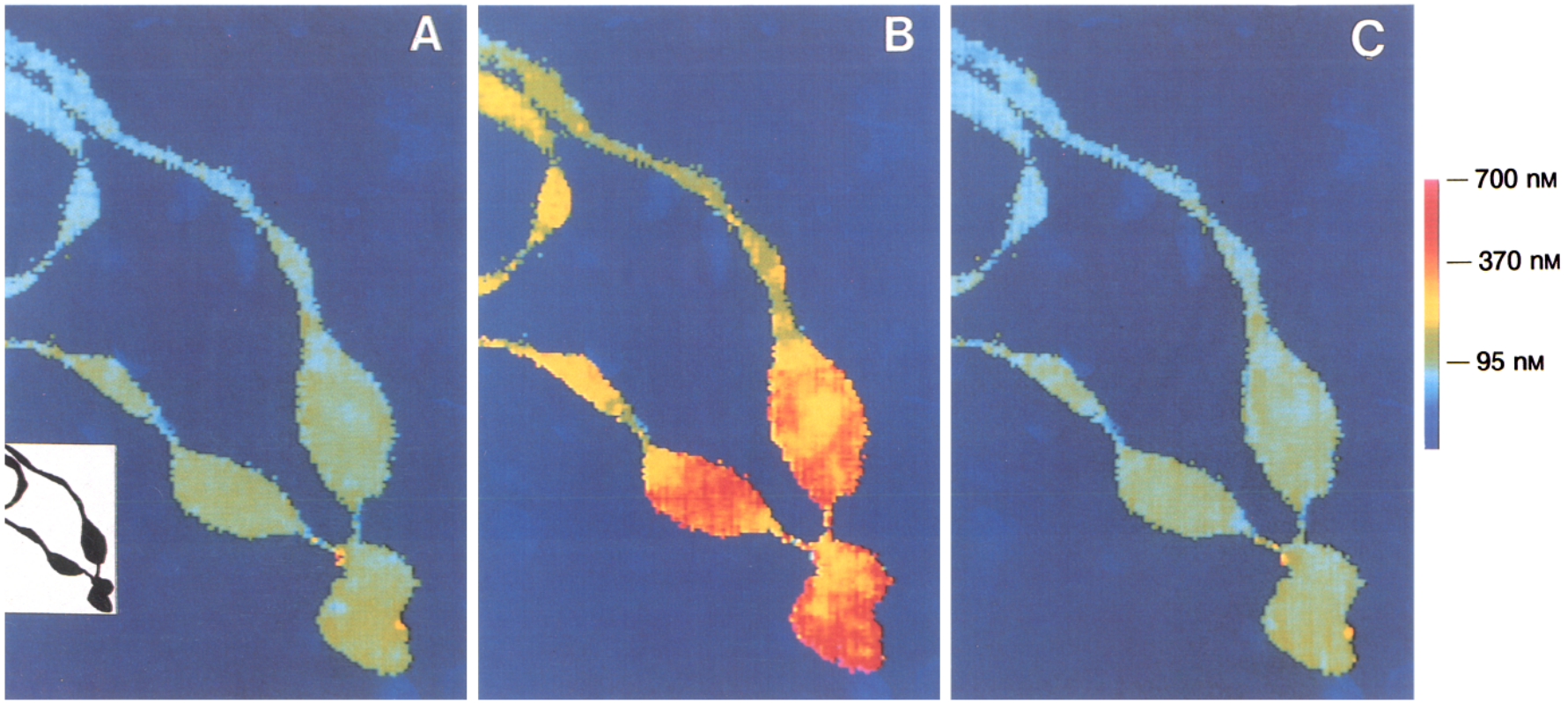

Figure 1. Growth cone calcium levels of neuron B5 increase during electrical activity. *Drawing at left* was traced from Nomarski image. *A,* In the absence of electrical activity, rest calcium levels are low in the 4 growth cones shown. *B,* When action potentials are evoked in the soma, calcium levels rise in growth cones and also in the neurites. *C,* Calcium levels return to their rest values shortly after action potentials are terminated. Note difference between calcium levels in growth cone and those in neurites in each panel. Image size, 140 × 270 pixels. *Color scale* on the right of this and subsequent figures indicates free calcium levels. Growth cones, approximately 20 μm wide.

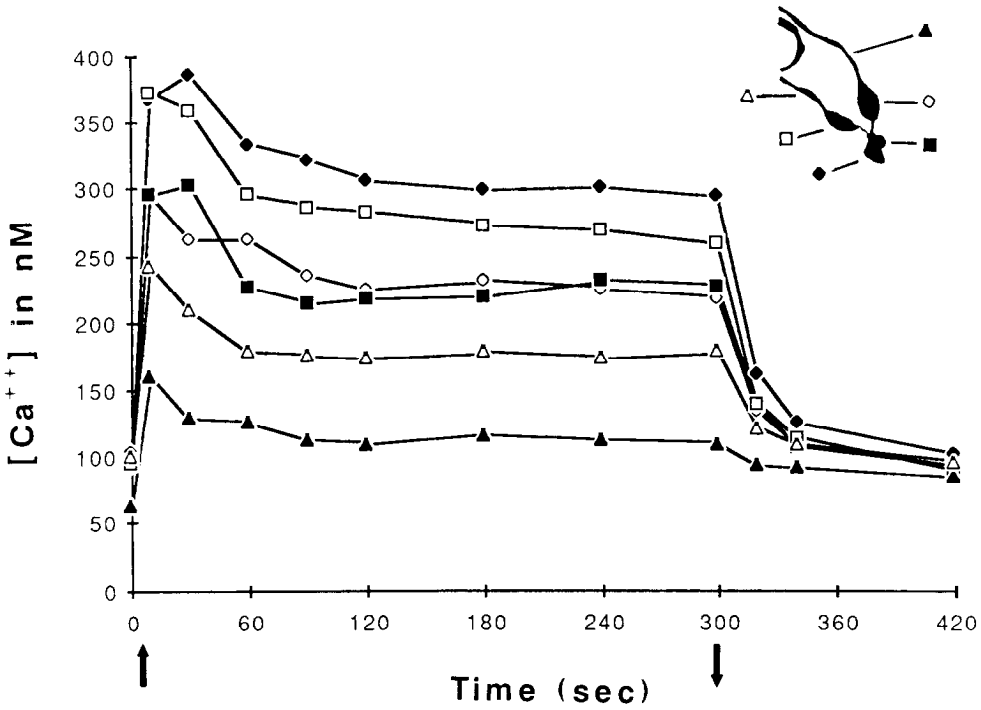

Figure 2. Temporal changes in calcium levels of neuron B5 during electrical activity. Calcium levels were monitored in 4 growth cones (*squares* and *diamonds*) and 2 neurites (*triangles*) shown in Figure 1 (*inset*). Action potentials were evoked at a frequency of 3/sec immediately after the reading at time zero and were sustained for 300 sec. Calcium levels quickly reached a plateau during the action potentials and then returned toward rest levels after action potentials stopped (*downward arrow*). Calcium levels rose to higher values in the growth cones than in either of the neurites.

enty percent of these neurons showed appreciable extension (5 μm or greater) during the course of observation. In 9 neurons, neurite morphology was observed quantitatively for time periods of 20 min or longer. These observations gave a mean outgrowth rate of 22.2 ± 0.16 μm/hr. This rate of extension was similar to rates for neurons that were not loaded with fura-2, indicating that the procedures had not adversely affected outgrowth. The remaining neurons in the group were not examined for a sufficient time to establish growth rates.

Effect of membrane potential on free calcium in growth cones

Intracellular microelectrodes were used to evoke action potentials in the somata of neurons B5, B19, and unidentified neurons plated in culture. Impalement of the cell body did not affect calcium levels in the neurites or growth cones as long as action potentials were not evoked. Action potentials generated in the cell body caused an abrupt rise in intracellular free calcium in the growth cones of all neurons we tested. In some neurons in which the indicator loading was adequate, as few as 4 action potentials caused an observable increase in calcium levels in growth cones and neurites. Increasing the number or frequency of action potentials caused larger increases in calcium levels. Figure 1 illustrates the changes evoked in 4 growth cones during a burst of 50 action potentials (5/sec for 10 sec). Data acquisition time occupied the last 2.5 sec of the spike train. The false color image of Figure 1*A* indicates that the rest levels of calcium were low (about 100 nM) and that a calcium gradient existed such that calcium levels were higher in the growth cone than in the neurite. During the spike train, calcium levels increased in the neurites and, to a greater extent, in the growth cones. Figure 1*C* was made 45 sec after the spike train was terminated and shows that recovery was nearly complete at that time.

To examine spatial and temporal changes in free calcium more precisely, we plotted calcium levels at 6 regions of the field shown in Figure 1 during a 5 min period in which action potentials were evoked continuously at 3/sec (Fig. 2). At this frequency, a plateau level of calcium was rapidly established and maintained for the duration of the spike train. Calcium levels in each of the growth cones remained similar throughout the train and were significantly higher than in the proximal neurites. During sustained trains of action potentials (4–5/sec) in neuron B19, the fura-2 fluorescence ratio reached levels of approximately 3 (461 nM calcium), and these levels were sustained for as long as the spike trains were maintained (durations up to 12 min were tested).

Where spike trains were limited to 45–60 sec duration, calcium levels were rapidly restored to rest values upon cessation of action potential activity (typically in less than 1 min). The recovery times measured here were in the same range as recovery times for calcium loads measured in somata of other molluscan neurons by arsenazo III absorbance (Ahmed and Connor, 1979; Gorman and Thomas, 1978; Smith and Zucker, 1980) and in crustacean neurites (Graubard and Ross, 1985). Where 5–10 min spiking protocols were employed, recovery sometimes required 3–4 min. Neuron B5, with its relatively broad action potential (20–25 msec), showed greater changes in calcium for a given number of action potentials at a given frequency than did neuron B10, which had a 2–4 msec action potential.

Figure 3 illustrates the cumulative effects of action potential train length in neuron B19, where growth cones were near the cell body. Records were taken after trains of 10 and 30 spikes, and show again that the most extreme changes occur in the growth cones. Here we found that the fluorescence ratio change recorded from the cell body was far smaller than that of neurites for a given spike train. Presumably this resulted from the larger calcium sink presented by the cell body.

We tested whether the rise in intracellular calcium during the generation of action potentials was caused by influx of calcium from the bathing solution by perfusing cobalt into the medium surrounding the growth cones. A puffer pipette filled with 10 mM cobalt chloride in culture medium was positioned adjacent to the growth cone. First, action potentials were evoked and the

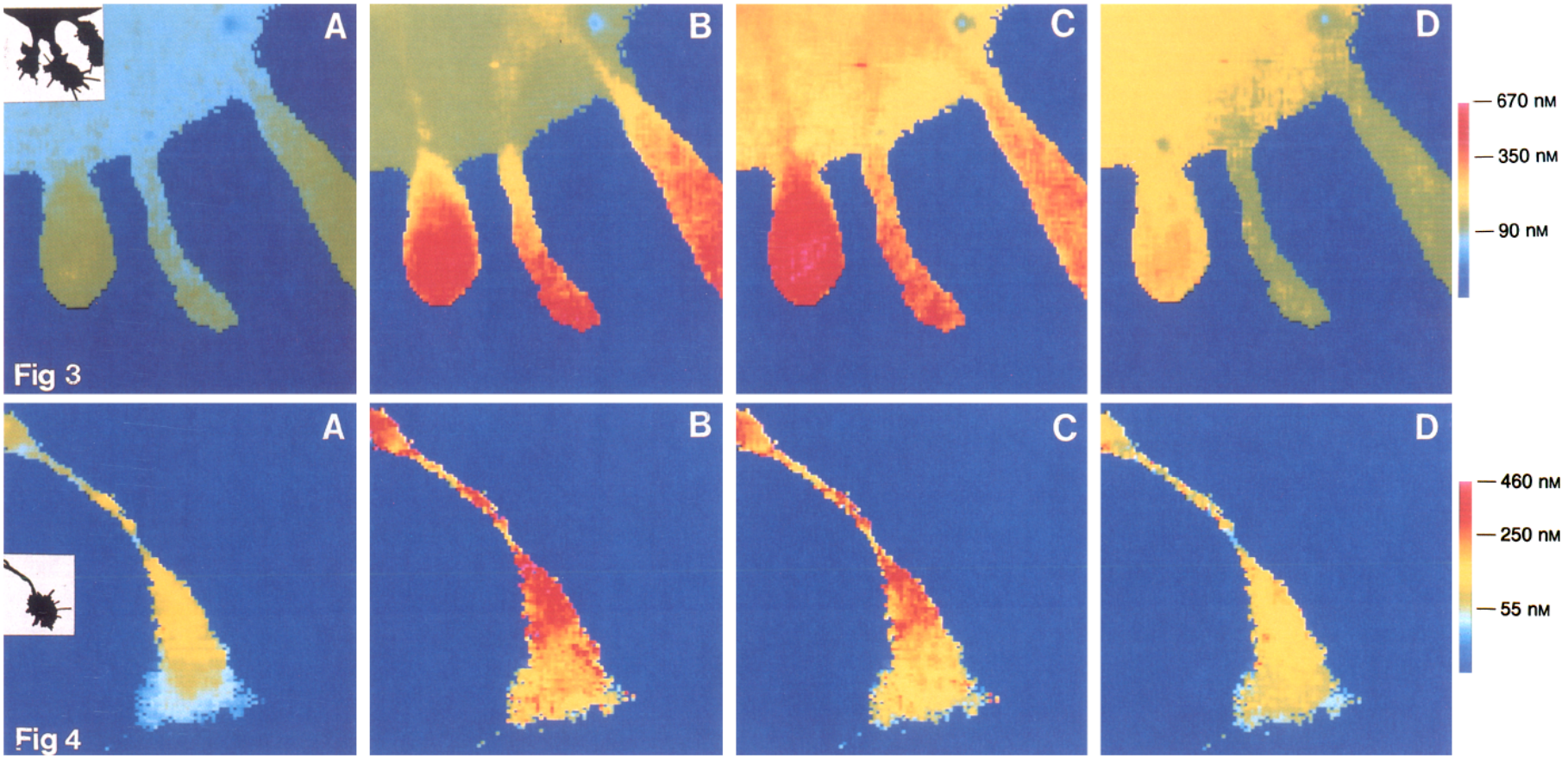

Figure 3. Changes in calcium levels in 3 growth cones near the soma of neuron B19 during electrical activity. *Inset* traced from Nomarski image shows growth cones and their filopodia as they emerge from the soma. *A,* Rest levels of calcium are low, but higher in the growth cones than in the soma. *B,* After 10 sec of action potential activity, calcium levels have increased in growth cones and, to a smaller extent, in the soma. Note that calcium levels have increased more in the leftmost growth cone than the other 2 growth cones. *C,* After 30 sec of activity, calcium levels continue to rise. Calcium levels have also increased in the soma. *D,* One min after activity was terminated, calcium levels have recovered to near rest values. Image size, 140 × 140 pixels.

Figure 4. Serotonin increases calcium levels in growth cones of neuron B19. *A,* Rest levels of calcium in growth cone whose Nomarski image is seen traced in *inset. B,* Calcium levels increased after focal application of serotonin from a pipette. *C,* One minute after serotonin pipette was removed from the medium, calcium levels in growth cone were still high. *D,* Calcium levels have decreased 10 min after serotonin application, but are still higher than at rest condition. Note regional differences in calcium levels within the growth cone. Image size, 140 × 140 pixels.

expected rise in intracellular calcium was observed. Then, cobalt was ejected into the region of the growth cone by applying positive pressure to the pipette. With cobalt present, the generation of action potentials in the cell body failed to give rise to increased intracellular calcium in the growth cone ($n = 6$). This indicates that the rise in intracellular calcium evoked by action potentials was caused by an increased calcium conductance at the growth cone membrane. Thus, action potentials, a signal known to inhibit growth cone motility, caused entry of calcium into the growth cone.

In a small number of cases ($n = 3$) we observed that hyperpolarization of the cell body caused a decrease in growth cone calcium levels. Thus, small changes in membrane potential at the soma can also affect calcium levels at the growth cones.

Increase in growth cone calcium evoked by serotonin

Serotonin was also shown to inhibit growth cone motility in *Helisoma* neurons (Haydon et al., 1984). However, unlike action potentials, which inhibit growth cone motility of all neurons that we have studied, serotonin selectively inhibits motility in the growth cones of neuron B19, but not those of neurons B4 and B5 (McCobb and Kater, 1985). The ability of serotonin to inhibit motility may be related to calcium changes it may evoke at the growth cone. We therefore tested to see whether serotonin changed free calcium levels selectively in these neurons by perfusing onto their growth cones.

Serotonin was puffed onto individual growth cones while calcium concentration was monitored. The precise concentration of serotonin at the growth cone was probably less than that in the pipette because of the dilution that occurred in the space between the pipette and the growth cone. Pipette concentrations of 10^{-5} M serotonin routinely evoked changes in calcium concentrations in growth cones of neuron B19 (Fig. 4). Such changes were evoked rapidly and had relatively long-lasting effects, presumably due to the persistence of serotonin in the surrounding medium. Even with brief applications for 5–10 sec, growth cones of neuron B19 showed elevated calcium concentrations that lasted for several minutes. In every case in which serotonin was puffed onto the growth cones of a neuron B19 ($n = 6$), there was significant elevation of intracellular calcium levels. Serotonin application was tested at 2 separate locations in each neuron, with similar results. Application of serotonin increased the fura-2 ratio from 1.31 ± 0.25 (125 nM calcium) to 2.41 ± 0.26 (330 nM calcium; $n = 10$). It is noteworthy that the effect of serotonin on growth cone calcium was spatially heterogeneous in neuron B19; areas that were higher in calcium before serotonin application remained higher after application (Fig. 4). Membrane potential was monitored in 4 of the 6 neurons during serotonin application. No action potentials were observed, nor were there other detectable voltage changes at the soma, except in one case where a 10 mV hyperpolarization was noted.

As serotonin has no effect on the motility of growth cones of neurons B4 and B5 (Haydon et al., 1984; D. P. McCobb and S. B. Kater, unpublished observations), we repeated the calcium-monitoring experiments with these neurons (Fig. 5). The calcium levels of growth cones of neurons B4 and B5 were unaffected by the application of serotonin, even at a concentration of 10^{-4} M ($n = 9$). The morphology of these growth cones remained unchanged, and even with repetitive puffs no increase in calcium concentration was observed, despite the fact that subsequent brief stimulation of action potentials from the cell body evoked a rapid and pronounced increase in intracellular calcium levels in the same growth cones (Fig. 5*C*). The results indicate that serotonin selectively increases calcium levels in growth cones of neuron B19 but not in neurons B4 and B5. These data parallel our previous findings of selective effects of serotonin on neurite outgrowth in neuron B19.

Additional observations demonstrated that non-neuronal cells may also respond selectively to serotonin. On several occasions we observed non-neuronal cells (as described by Wong et al., 1981) in the immediate vicinity of growth cones that were under study. During the application of serotonin to the bath, some non-neuronal cells displayed substantial increases in intracellular calcium (Fig. 6). However, as with growth cones, not all non-neuronal cells responded to serotonin; calcium levels in some cells remained unchanged. Thus, non-neuronal cells can also respond selectively to the presence of neurotransmitters. While this finding was not a primary aim of this study, it may indicate that processes such as movement in these highly motile cells might be under similar regulation to that of growth cones.

Free calcium in growth cones that spontaneously stopped growing

Soon after plating in cell culture, *Helisoma* neurons develop growth cones and extend neurites. However, these growth cones progress (over a period of 4–7 d) to a nonmotile state in which neurites no longer elongate. These growth states can be distinguished morphologically. Motile growth cones are broad, flat, and phase-dark, whereas nonmotile growth cones are club-shaped and phase-bright. This gave us the opportunity to compare calcium levels in growth cones that spontaneously stopped growing with calcium levels in growth cones that were experimentally treated to inhibit growth.

We found a large difference in resting calcium levels in motile as compared to nonmotile growth cones, as shown in Table 1. Calcium levels in motile growth cones averaged 2.3 times higher than levels in nonmotile growth cones. This difference in resting calcium levels in growing and nongrowing neurons is consistent with findings on mammalian neurons in culture (Connor, 1986).

We also found regional variation in calcium levels within *Helisoma* neurons that had motile growth cones. The calcium level in the soma and proximal neurites was uniformly low in all the neurons we examined. However, calcium levels were significantly higher in the growth cones. The mean fura-2 ratio for growth cones was 1.50 ± 0.05 (157 nM calcium), whereas the mean ratio for neurites of the same neurons was 1.19 ± 0.07 (105 nM calcium; $p < 0.002$ for 16 growth cones on 13 neurons. Note that this data set is different from that used for Table 1). Figure 7 demonstrates a typical difference between neurites located near the cell body (*E*) and near growing growth cones (*A, D*). Calcium levels along the neurite were higher near the growth cone than near the soma, resulting in a calcium gradient. There were no consistent differences in resting calcium distributions between neurons B5, B19, or any of the unidentified neurons plated in the cultures. In contrast to these data, neurons with nonmotile growth cones had much lower resting levels of calcium and never showed consistent regional differences in calcium.

In addition to regional variation we also observed spontaneous temporal changes in calcium levels in growth cones (Fig. 7). The calcium level increased as a growth cone moved across the substrate during a 1 hr observation period (Fig. 7, *A–C*), but was always higher than proximal neurites. Furthermore, a second growth cone on the same neuron (Fig. 7*D*) showed higher

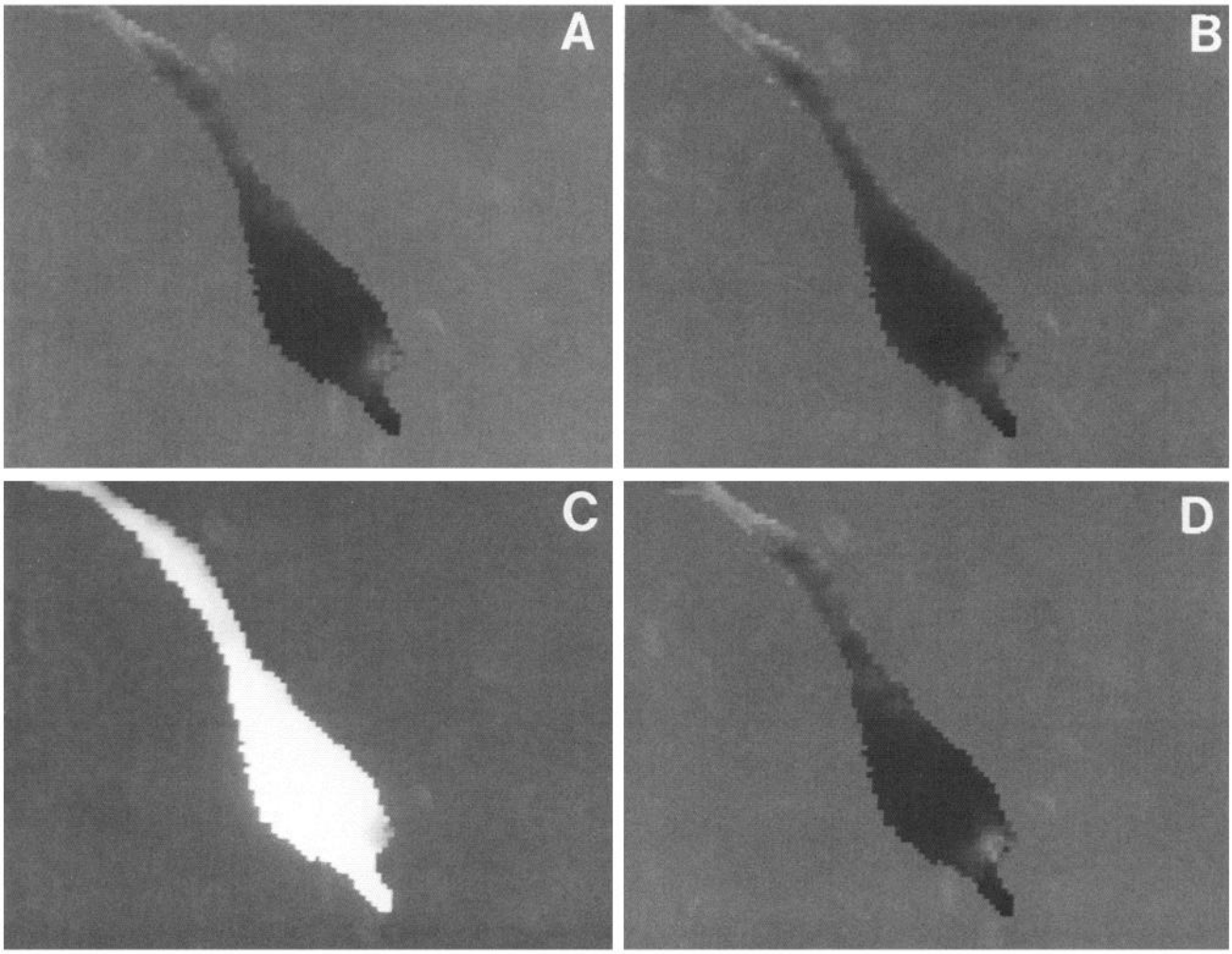

Figure 5. Serotonin has no effect on calcium levels in growth cones of neuron B5. *A,* The normally low rest level of calcium remains low (*B*) after serotonin is applied to the growth cone. *C,* Calcium levels increase after action potentials are evoked in the soma. *D,* Calcium levels recover to rest levels after action potentials are terminated. Image size, 140 × 140 pixels.

levels of calcium than the growth cone of Figure 7*A*, monitored at approximately the same time. Thus, different growth cones on the same neuron could simultaneously show different concentrations of calcium. The largest differences were generally observed between growth cones separated by several branch points or on different sides of the soma.

Comparison of bath-applied to injected fura-2

Incomplete de-esterification and compartmentalization of fura-2 are 2 potential problems in using the /AM form of the dye to estimate absolute levels of free calcium in cells (Almers and Neher, 1985; Connor, 1986). In order to assess the accuracy of our measurements of /AM-loaded neurons, we therefore compared our results with those from neurons that had been directly injected with the ionic form of fura-2. Table 1 summarizes observations comparing the 2 methods of indicator loading in growing and nongrowing neurons. Within each group there was no significant difference in resting calcium levels for /AM-loaded or injected neurons. Also, there were no discernible organellar compartments in the *Helisoma* neurites that trapped the indicator and showed markedly different calcium levels from the rest of the cytoplasm. Such traps are not uncommon in mammalian neurons in culture (J. A. Connor, unpublished observations). The resting calcium level for *Helisoma* growth cones (Table 1) compares well with resting calcium levels in other molluscan neurons (30–100 nM) measured by other techniques (Ahmed and Connor, 1979; Dipolo et al., 1983; Requena et al., 1984).

The saturation spiking data of Table 1 were obtained by evoking action potentials at 10–15 Hz for 8 or more sec, as this produced a maximal plateau response in these neurons. We did not make a large number of measurements of this sort because of the retarding activity of action potentials on outgrowth (Cohan and Kater, 1986). The maximum values of fluorescence ratios for injected cells were somewhat larger than were those for the /AM-loaded cells. This may have resulted from a small percentage of the /AM-loaded dye's either not being completely hydrolyzed or being trapped in compartments that did not undergo calcium changes with electrical activity. The 380 nm excited fluorescence of fura-2 became quite small at high calcium levels, and a small amount of nonreporting indicator could bias the estimation of large calcium changes to the low side. With this reservation, it is probably safe to conclude that *Helisoma* neurons do not have the problems associated with the use

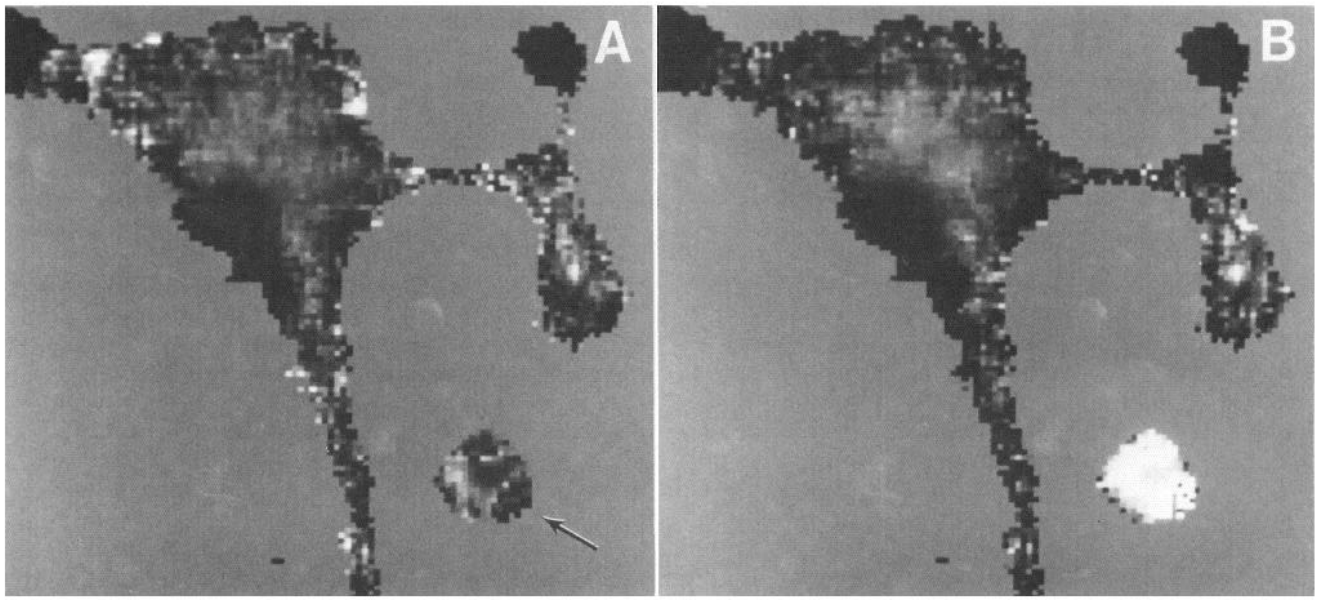

Figure 6. Serotonin affects calcium levels in non-neuronal cells. Rest calcium levels in non-neuronal cell (*arrow, A*) increase after exposure to serotonin (*B*). Image size, 140 × 140 pixels.

of fura-2 as a cytoplasmic calcium indicator that have been encountered elsewhere (Almers and Neher, 1985; Williams et al., 1985).

Discussion

In the present report we used the calcium indicator fura-2 to compare intracellular levels of free calcium in growth cones of isolated *Helisoma* neurons under a variety of experimental conditions. Our aim was to monitor changes in calcium levels in growth cones under conditions known to inhibit growth cone motility. We found that electrical stimulation of the cell body caused a rise in calcium levels in the growth cones of all neurons tested. With brief stimulation periods, calcium levels quickly recovered to normal values, whereas longer stimulation periods required longer recovery times. Focal application of serotonin to growth cones also increased intracellular calcium. This response was specific for neuron B19 and did not occur when serotonin was applied to neurons B4 or B5, just as was shown for inhibition of motility in these neurons (Haydon et al., 1984; McCobb and Kater, 1986). In comparisons of growing growth cones with growth cones that had spontaneously stopped growing, we found that motile growth cones had higher free calcium levels than did non-motile growth cones. Furthermore, the distribution of calcium in neurons that contained growing growth cones was heterogeneous; calcium levels were always higher in the growth cone than in the neurite or soma. Free calcium levels also changed dynamically as growth cones advanced over their substrate. Table 2 summarizes these data for neurons B4, B5, and B19. The data indicate that calcium levels in growth cones are modulated by electrical and chemical signals that inhibit growth cone motility, and that calcium levels in growth cones vary in different states of outgrowth.

Ionic currents mediated by calcium in growth cones have been the subject of recent investigations. Measurements with voltage-sensitive dyes (Grinvald and Farber, 1981) and patch recordings (Anglister et al., 1982) have suggested the presence of active ionic currents at the growth cones of neuroblastoma cells. The regenerating tips of severed axons have been shown to exhibit calcium-dependent action potentials in cockroach (Meiri et al., 1981), and barium-dependent action potentials in lamprey (MacVicar and Llinás, 1985). Recently, arsenazo III was used to measure influx of calcium in soma, neurites, and growth cones

Table 1. Comparison of calcium levels in motile and nonmotile growth cones and with 2 loading methods

	/AM loaded		Injected	
	Motile	Nonmotile	Motile	Nonmotile
Resting levels in growth cones	(130)	(56)	(125)	(44)
	1.34 ± 0.19	0.88 ± 0.18	1.31 ± 0.11	0.80 ± 0.13
	$n = 13$	$n = 9$	$n = 6$	$n = 4$
Max F_{340}/F_{380} ratio during saturation spiking	(703)		(795)	
	3.91 ± 0.23		4.21 ± 0.62	
	$n = 4$		$n = 5$	

Nonmotile growth cones have significantly lower calcium levels than motile growth cones. The means and standard errors for fura-2 fluorescence ratios are shown and the calculated calcium concentrations appear in parentheses. Calcium levels are also compared in neurons in which fura-2 was either bath applied (/AM form) or directly injected into the soma. Peak calcium levels obtained during periods of sustained spiking are also shown.

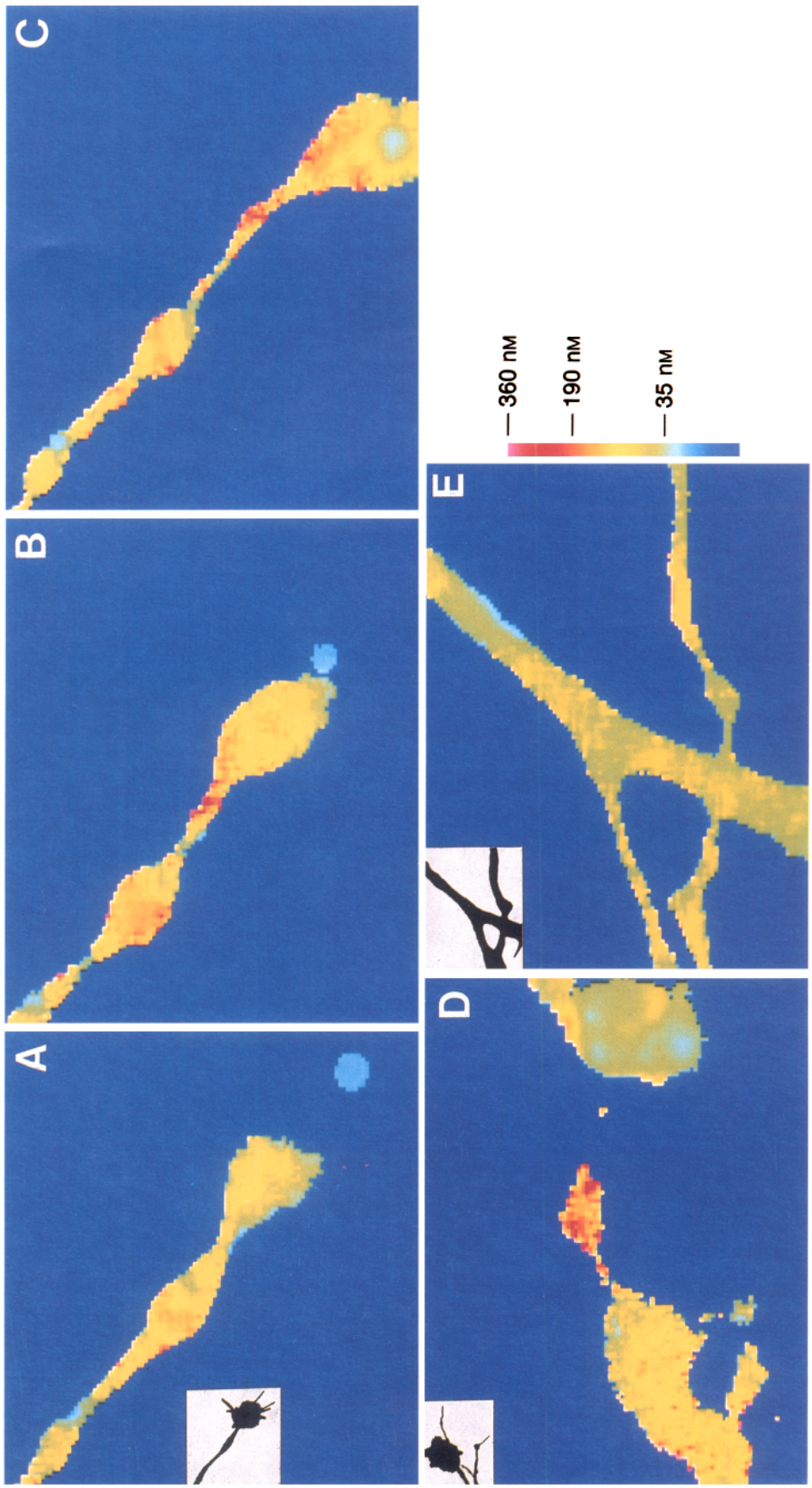

Figure 7. Spontaneous changes in calcium levels in growth cones of neuron B19 during outgrowth. *A–C*, Position of a growth cone at 0, 30, and 64 min of observation, respectively. As growth cone (*traced image, inset*) advances across substrate, calcium levels spontaneously fluctuate. Note advance of growth cone in relation to small stationary particle (*blue sphere*) on substrate. *D*, Another growth cone (*traced image, inset*) from the same neuron shows a higher calcium level than the growth cone in *A*. Both photos taken within 1 min of each other. *E*, Calcium levels in neurites (*traced image, inset*) are usually lower than levels in growth cones (compare *A–D* with *E*). Image size, 140 × 140 pixels.

of neuroblastoma cells (Bolsover and Spector, 1986). Recordings from *Aplysia* growth cones (Belardetti et al., 1986), however, have not demonstrated significant macroscopic calcium currents, although some single-channel calcium currents were observed. Whether calcium channels appear in these growth cones at a later time is unknown. Nonetheless, growth cones in widely varying species appear to support action potentials that result in the influx of calcium into the intracellular compartment.

Our finding that hyperpolarization of the cell body changed calcium levels within the growth cones suggests the presence of a steady-state calcium current in the growth cone that is modulated by membrane potential. Steady-state calcium currents have also been detected in the growth cones of retinal explants (Freeman et al., 1985). These findings further indicate that space constants for these neurons are sufficiently large to influence growth cones at the ends of neurites via electrical events in the soma. Thus, synaptic activity that does not reach threshold may also be able to regulate growth cone movements. Changes in growth cone calcium, induced by minor variations in membrane potential, may be directly related to changes in growth cone motility.

The observation that calcium levels were higher in motile, as opposed to nonmotile, growth cones has also been made by Connor (1986). Calcium levels were also higher in the growth cone than in the soma or neurite, and may result from the higher density of calcium channels thought to exist in the growth cone membrane (Bolsover and Spector, 1986). Such localized regions of high free calcium may be important functionally in motile behaviors of the growth cone. This hypothesis is supported by studies on neutrophils, in which calcium levels were highest in the lamellipodia of migrating cells and pseudopods of ingesting cells (Sawyer et al., 1985). The localization of calcium-sequestering mechanisms could provide a means for producing spatially heterogeneous calcium levels during critical periods of neuronal development.

Calcium has been suggested as having a regulatory role in the motility of a wide variety of cell types. Much of the evidence for this was based originally on studies of contractility, in which calcium binding to the protein troponin regulates the interaction of actin and myosin filaments (Mannherz and Goody, 1976). Calcium is also known to control ciliary beating (Kung and Saimi, 1982), the disassembly of actin and myosin filaments (Allen and Taylor, 1975; Schliwa et al., 1981), the movement of particles during axonal transport (Brady et al., 1985), and the activity of a variety of proteins (Schliwa, 1981). Using the calcium indicator quin2, it has recently been shown that calcium levels are elevated in neutrophils during migration and phagocytosis (Sawyer et al., 1985). Elevated calcium levels are also associated with the turning response of growth cones evoked by application of nerve growth factor to cultured dorsal root neurons (Gunderson and Barrett, 1980). Calcium has been proposed to regulate axoplasmic fluidity and thereby influence growth cone motility (Llinás, 1980). Calcium channel blockers have been shown to reversibly inhibit locomotion of keratocytes (Cooper and Schliwa, 1985). Thus, calcium appears to be important in the motile responses of many cells.

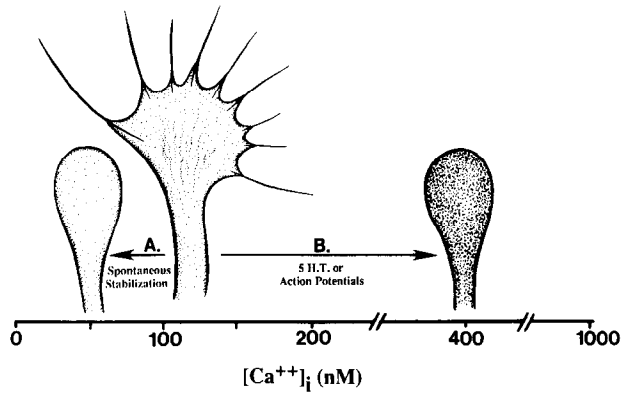

Figure 8. Summary diagram suggesting relationship between calcium levels and growth state of growth cones. The degree of *stippling* corresponds to the level of free calcium. Outgrowth is correlated with an optimal level of intracellular calcium, whereas much higher or lower calcium levels may cause cessation of outgrowth. Calcium levels in growth cones that have stopped growing as a result of action potentials or serotonin may eventually decrease to the low levels of growth cones that have spontaneously stopped growing.

Growth cone motility may depend quantitatively on intracellular calcium levels (Fig. 8). Compared to growth cones that have spontaneously stopped growing, somewhat elevated calcium levels, estimated here to be between 100 and 300 nM, are correlated with outgrowth, whereas much higher calcium levels, estimated here to be greater than 400 nM, are correlated with stimuli that inhibit outgrowth. The time course of intracellular calcium changes as neurons spontaneously progress from a growing to a nongrowing state is presently unknown. During the transition from growing to nongrowing states, we speculate that calcium levels in growth cones may increase to values similar to those for growth cones exposed to serotonin or action potentials. Subsequently, calcium levels may decline to those ob-

Table 2. Calcium levels and signals that affect growth cone motility

Identified neuron	Rest Ca	Ca level after APs	Outgrowth after action potential[a]	Ca level after serotonin	Outgrowth after serotonin[a]
4	+	++++	?	+	yes
5	+	++++	no	+	yes
19	+	++++	no	++++	no

Action potentials and serotonin selectively alter calcium levels in identified neurons and this is correlated with inhibition of neurite outgrowth. Action potentials caused an increase in growth cone calcium in 3 different types of identified neurons. Serotonin selectively caused an increase in growth cone calcium in neuron B19 but not neurons B4 or B5. These changes in calcium levels parallel the inhibition of growth cone motility and neurite outgrowth previously demonstrated for these 2 signals. The + symbols are intended to indicate diagrammatically the quantitative changes in calcium levels produced by action potentials and serotonin.

[a] Data from Cohan and Kater (1986) and Haydon et al. (1984).

served here, possibly as a result of a decrease in a steady-state calcium influx or an increased calcium-sequestering mechanism within growth cones. Thus, our data indicate that an optimum concentration of calcium within the growth cone is necessary to support outgrowth. Calcium levels much higher or much lower than this optimal level are correlated with cessation of outgrowth.

Recent experiments in which calcium levels have been manipulated in cultured *Helisoma* neurons (Mattson and Kater, 1987) support our findings. Calcium ionophores that would raise calcium levels to high values inhibit neurite outgrowth, as was shown for electrical activity and serotonin application. High calcium concentrations have also been shown to inhibit outgrowth in other molluscan neurons (Kostenko et al., 1983). Calcium channel blockers at low concentrations permit neurite elongation. However, at high concentration, these blockers inhibit elongation, indicating that low intracellular calcium levels do not support outgrowth, just as we have suggested for neurons that have spontaneously stopped growing.

Our observations could be explained by 2 different processes that might regulate both the maintenance and inhibition of motility. These processes could have activation kinetics that depend on 2 different concentrations of calcium. Activation of events necessary for growth cone motility and neurite outgrowth might require moderate levels of calcium, whereas activation of events necessary to inhibit motility might require considerably higher calcium levels. Thus the same messenger, calcium, could regulate both the initiation and the cessation of growth cone movement in a concentration-dependent fashion.

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