Regulation of Neuronal Growth Cone Filopodia by Intracellular Calcium

Vincent Rehder and S. B. Kater

Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado 80523

Filopodia have been regarded as the sensory extensions of neuronal growth cones. As such, filopodia assay distant environments and are important for directing growth cones toward their targets. Since the territory encountered by a growth cone depends on the area spanned by the filopodia, changes in filopodial length or number result in the "exploration" of different-sized regions of the environment. The present study tests the potential regulatory role of intracellular calcium levels ($[Ca^{2+}]_i$) on filopodial morphology in identified neurons from the snail *Helisoma*. Experimentally evoked changes in $[Ca^{2+}]_i$ were measured with the fluorescent calcium indicator fura-2 and directly correlated with growth cone filopodial morphology. A rise in $[Ca^{2+}]_i$ caused two distinct, concentration-dependent effects separable by their different time courses: within the first 10 min, filopodia underwent significant elongation, while the second phase was characterized by a massive loss of filopodia. Both of these behaviors were increased in a calcium-dependent fashion. The magnitude of both filopodial elongation and filopodial loss correlated well with the transient peak values of $[Ca^{2+}]_i$ reached during a given experimental treatment ($r \leq 0.98$). In addition to the direct effect of the initial transient rise in $[Ca^{2+}]_i$, there is evidence for a form of adaptation of filopodial behavior to sustained calcium levels. A transient change in $[Ca^{2+}]_i$ of as little as 30–50 nM reliably altered filopodial morphology. These results indicate that even small changes in intrinsic calcium homeostatic properties or extrinsic signals that alter intracellular calcium levels can act as regulators of the size of the environment sampled by an elongating growth cone.

During development neuronal growth cones traverse a complex environment to arrive at their final target. The prominent filopodia on growth cones are typically arranged in a fanlike fashion and have been proposed to play a role as chemical and tactile probes of the environment on the basis of experiments performed both *in vivo* and *in vitro* (Harrison, 1910; Nakai and Kawasaki, 1959; Sperry, 1963; Bentley and Toroian-Raymond,

1986; Hammarback and Letourneau, 1986; Bandtlow et al., 1990). This fanlike filopodial configuration may allow a growth cone to cover a vastly enhanced "search" area, as compared to the body of the growth cone itself, and would enhance the likelihood of encountering relevant environmental cues.

Available evidence suggests that filopodia are important in directing outgrowth *in vitro* (Letourneau, 1979; Bray and Chapman, 1985; Hammarback and Letourneau, 1986; Goldberg and Burmeister, 1989) and possibly for steering in response to guidance cues *in vivo* (Bentley and Keshishian, 1982; Taghert et al., 1982; Bentley and Toroian-Raymond, 1986; O'Connor et al., 1990). An example of the functional importance of growth cone filopodia is provided by the *in vivo* preparation of the grasshopper limb bud. Bentley and Toroian-Raymond (1986) observed that growth cones devoid of filopodia continued to grow, though often along aberrant routes, suggesting a loss of steering capability. Filopodia in this system can have a length exceeding 50 μm and may physically bridge the distance between different guidepost cells, thereby possibly causing redirection of growth during pathfinding (Ho and Goodman, 1982; Taghert et al., 1982; Bentley and Caudy, 1983; O'Connor et al., 1990). Another example of the functional importance of filopodia *in vivo* was reported by Bandtlow et al. (1990). Video time-lapse studies of dorsal root ganglion (DRG) growth cones revealed that the contact of a single filopodium with a mature oligodendrocyte was sufficient to trigger arrest and often collapse of the growth cone. A striking example of the guiding role of filopodia comes from Hammarback and Letourneau (1986), who demonstrated that growth cones *in vitro* could quickly traverse a gap of low-adhesivity substrate when this gap was spanned by even a single filopodium establishing contact on a distant high-adhesivity substrate. In such cases, information obtained by a single filopodium could result in a redirection of growth.

Studies *in vivo* (Roberts and Taylor, 1983; Taylor and Roberts, 1983; Tosney and Landmesser, 1985; Caudy and Bentley, 1986) and *in vitro* (Ludueña, 1973; Letourneau, 1979; Bray and Chapman, 1985) convincingly demonstrate that the shape of growth cones and its number of filopodia depend on the environment they traverse, with growth cones exhibiting a more complicated morphology at "decision points" (Taghert et al., 1982; Raper et al., 1983; Tosney and Landmesser, 1985; Eisen et al., 1986; Holt, 1989). On the basis of such evidence, it seems plausible that physiological control over the number of filopodia, as well as filopodial length, could allow a growth cone to sample information from different-sized regions of the environment. In fact, a variety of stimuli have been shown to modify filopodial number and length. Using a simplified model system *in vitro*, Gundersen and Barrett (1980) reported that the number

of filopodia increases on the side of a localized application of NGF. A rise in cAMP induced by forskolin can cause a reduction in filopodial number and growth rate (Mattson et al., 1988a). Electric DC fields, applied across growth cones, cause growth toward the cathode (Jaffe and Poo, 1979; McCaig, 1986, 1989), with a higher density of filopodia directed toward the cathode than the anode (McCaig, 1986).

With the large growth cones of neurons from the snail *Helisoma*, it has been possible to analyze not only the environmental messengers altering growth cone behavior, but also the role of the second messenger calcium. Application of 5-HT was shown to cause a neuron-specific withdrawal of filopodia and, ultimately, cessation of outgrowth (Haydon et al., 1984). 5-HT was then demonstrated to bring about its effects through a rise in intracellular calcium (Cohan et al., 1987). Direct application of the calcium ionophore A23187, qualitatively, was also reported to induce filopodial loss (Mattson and Kater, 1987). Additionally, changes in growth rates and in filopodial numbers were also evoked by electrical stimulation (Cohan and Kater, 1986; Cohan, 1990), a treatment known to raise the intracellular calcium concentration (Cohan et al., 1987). Taken together, these earlier studies proposed a link between a rise in intracellular calcium and the status of filopodia. The present study provides a quantitative analysis of the relationship of intracellular calcium and filopodial behavior and reveals three new aspects of the control of growth cone filopodia. In a dose-dependent fashion, a rise in intracellular calcium is related to the following, sequentially occurring, highly stereotyped, behaviors: (1) filopodial elongation, (2) filopodial loss, and (3) filopodial adaptation. This series of events could significantly affect the sensory capabilities of neuronal growth cones.

Materials and Methods

Establishing cultures of identified neurons. Adult snails of an inbred laboratory stock of the snail *Helisoma trivolvis* were used for all experiments (Haydon et al., 1985; Kater and Mattson, 1988). Buccal ganglia were dissected, and identified neurons B5 were individually removed and plated onto polylysine (MW, 70–150 K; Sigma, St. Louis, MO)-coated glass coverslip–bottomed cell culture dishes (Falcon 1008, 35 mm). Neurons were grown in defined medium consisting of half-strength Leibovitz L-15 from which all inorganic salts were deleted (special order, GIBCO, Grand Island, NY). Salts were subsequently added to a final concentration of 40 mM NaCl, 1.7 mM KCl, 1.5 mM MgCl₂, 4.1 mM CaCl₂, 5 mM HEPES, 50 μg/ml gentamycin, and 0.15 mg/ml glutamine, pH 7.3, in distilled water. Cell culture dishes contained 1 ml of conditioned medium to promote neurite outgrowth (Wong et al., 1981, 1984). Neuronal cultures were kept in a humidified chamber at room temperature (18–22°C) and were used 20–26 hr after plating in order to obtain growth cones of comparable age and size that exhibited mature veils and filopodia.

Treatment of neuronal cultures. To alter intracellular calcium levels within neurons, the following substances and media were employed. (1) Calcium-free medium contained the concentrations given for defined medium above except that MgCl₂ was substituted for CaCl₂ and 0.5 mM EGTA was added. (2) To depolarize neurons, media of varying extracellular potassium concentrations (8.5 mM, 17 mM, and 34 mM) were made by substituting KCl for NaCl (8.5 and 17 mM) or by adding an additional 17 mM KCl to the 17 mM medium to obtain 34 mM KCl. A full substitution of 34 mM KCl for NaCl would have led to a substantial reduction in NaCl (6 mM) and was therefore avoided. To test for possible osmotic effects introduced by the addition of 17 mM KCl, controls were performed introducing additions of 17 mM NaCl or 34 mM sucrose. These additions had an effect neither on [Ca²⁺]ᵢ nor on the filopodial number (not shown). (3) The calcium ionophore 4-bromo A23187 (Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide (DMSO; Aldrich, Milwaukee, WI) as a 0.1 mM stock solution. The ionophore was added in 15 μl volumes to the periphery of a 2 ml medium–con-

taining dish to obtain a final concentration of 0.75 μM ionophore. The medium subsequently was mixed by gentle agitation with a Pasteur pipette. To test for possible effects of the carrier, 15 μl of DMSO (0.75% final concentration) were added to the dish in identical manner as the ionophore. Administration of the carrier alone had no effect on [Ca²⁺]ᵢ or the number of filopodia. Medium changes were performed by carefully removing all medium from the dish, except from the center coverslip well, which contained about 200 μl of medium. New medium was introduced with three to four washes. To control for the effect of a medium change on [Ca²⁺]ᵢ or the number of filopodia, growth cones growing in normal medium were rinsed four times with normal medium; the medium change alone had no effect on [Ca²⁺]ᵢ or filopodial number.

Isolation of growth cones. Growth cones were isolated by transecting neurites close to the growth cone proper with a glass micropipette attached to a micromanipulator (see Rehder et al., 1991). Experiments on isolated growth cones were performed 60 min after transection on neurons that had previously been filled with fura-2. This waiting period proved sufficient to allow isolated growth cones to restore their [Ca²⁺]ᵢ to stable values after the transient transection-induced increase.

Calcium measurements. Somata of neurons were filled by microelectrode impalement with fura-2 pentapotassium salt (10 mM in H₂O; Molecular Probes) by iontophoresis and used 45–60 min after the injection. The distribution of free intracellular calcium within a growth cone before and after treatments was homogeneous with this method of loading. Therefore, calcium measurements were representatively taken from the central region of the growth cone. Cultures were viewed with an intensified CCD camera (Quantex, Santa Clara, CA) on an inverted microscope (Zeiss, ICM 405; Nikon Fluor 40×/1.3 oil objective). The camera output was digitized with a QX7-210 image processing system (Quantex) and converted into a 640 × 480 pixel image. Images taken at two different excitation wavelengths (350 ± 10 nm and 380 ± 10 nm interference filters) were each averaged for 16 frames (540 msec). The fluorescence emission was filtered with a 495 nm long-pass emission filter. Depending on the brightness of the signal, neutral density filters were used in the excitation pathway to minimize bleaching of the dye and damage to the cell. The ratio (R) of the fluorescence intensity of the two images taken at 350 nm and 380 nm was converted to obtain an estimation of calcium concentrations using the formula [Ca²⁺]ᵢ = $K_d[(R - R_{min})/(R_{max} - R)](F_0/F_s)$ according to Grynkiewicz et al. (1985). The values determined for our system are: R_{min}, 0.48; R_{max}, 13.25; F_0/F_s, 10; K_d, 224.

Data acquisition and statistics. Analysis of filopodial behavior was performed on unloaded neurons, and correlated to [Ca²⁺]ᵢ obtained from parallel experiments on fura-2 loaded neurons. This approach was taken in order to eliminate two potential complications: the introduction of a calcium buffer into the neuron, and the possibly adverse effects of repeated illumination of fura-2 during imaging of filopodial behavior. As many as 20 repeated viewings of fura-2 loaded cells had no effect on levels of intracellular calcium. To assess filopodial numbers, 35 mm negatives of growth cones were taken through a 40× objective (Zeiss, Neofluar 40/0.75) on an inverted microscope (Zeiss ICM 405) prior to the treatment and at defined intervals thereafter. Negatives were viewed by projection and filopodia counted for each growth cone and time point. Since filopodial number depends, in part, on the size of a growth cone, a quantification by the criterion "number of filopodia/growth cone" would be strongly biased if a range of growth cones of different sizes were to be used. We therefore normalized the filopodia number to percentage of control. In control experiments, this value was assessed before and after 1 hr of control growth and found to be not significantly different, indicating that growth cones over this time interval did not grow larger and did not develop more filopodia. Only those growth cones that did not branch or fasciculate with other neurites during the experiment were used. Filopodial length was assessed by digitizing phase-contrast images of growth cones on a computer (Macintosh IIx) and using the software package IMAGE (W. Rasband, NIH). For this purpose, the length of all filopodia on a given growth cone was measured, regardless of whether these were extending, retracting, or not changing at all. Normalized data from growth cones were pooled, and expressed as mean ± SEM. Both the total number of growth cones and the number of cells used for each experiment are provided in the text. Since the number of growth cones (n) at different time points varied in some experiments due to missing values, n is given as, for example, n = 21–26 (indicating that at least 21 growth cones were measured at all time points and up to 26 at some time points). Overall differences between groups were determined performing an analysis of variance employing
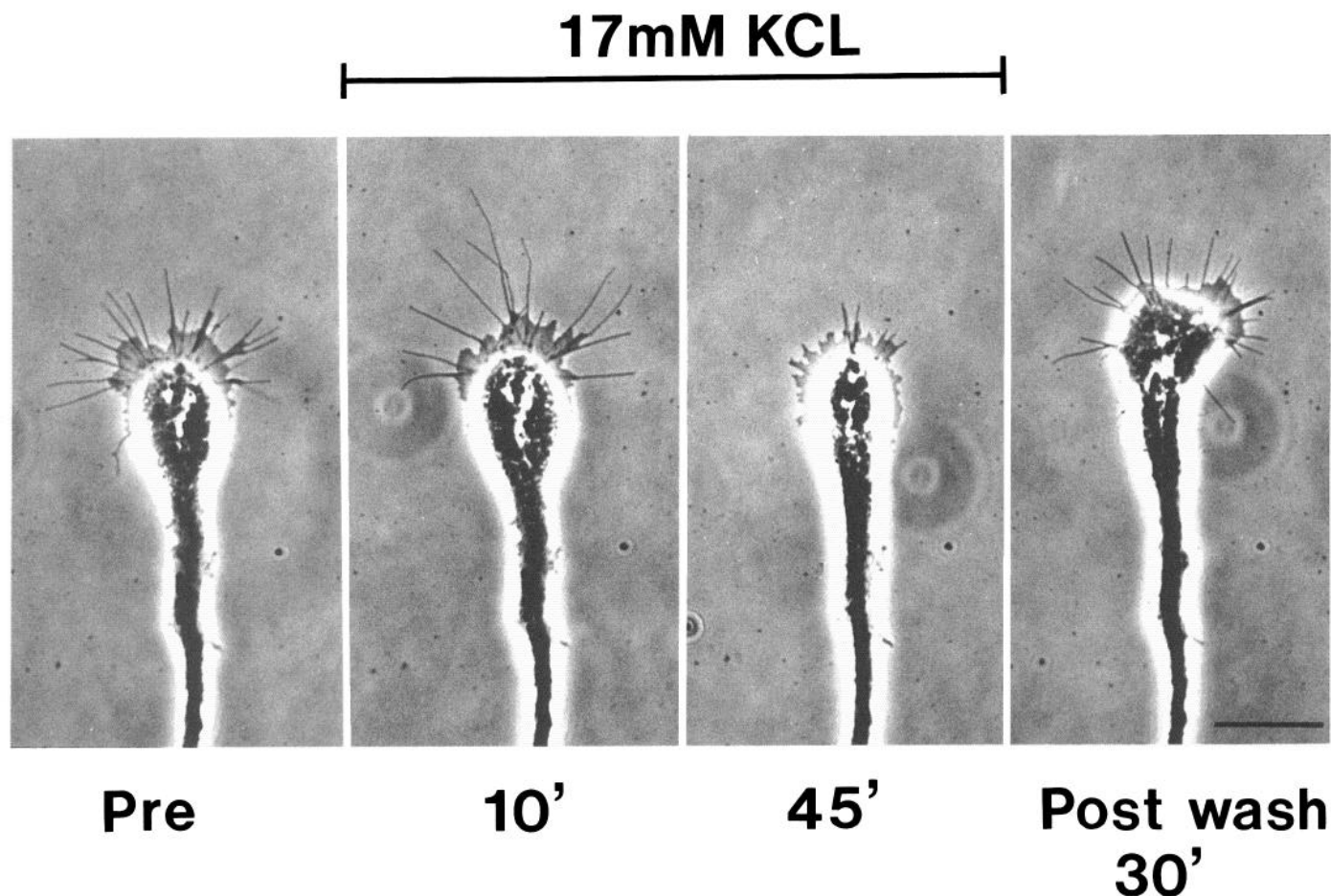
17mM KCL

Pre   **10'**   **45'**   **Post wash 30'**

Figure 1. Phase-contrast micrographs demonstrating the effect of a 10-fold elevation of extracellular [K⁺] (from 1.7 to 17 mM) on filopodial length and filopodial number. Growth cones of a neuron B5 display numerous filopodia and fully developed veil prior to the treatment (*Pre*). Within the first 10 min, several filopodia elongated significantly (*10'*). In a second phase, filopodia shortened and many were lost (*45'*). Return to normal medium completely reversed these effects: the lamellipodium reappeared, new filopodia were generated, and the filopodial number reached pretreatment levels (*Postwash 30'*). The *bar* indicates the presence of elevated $[K^+]_o$. Scale bar, 20 μm.

the procedure GLM (General Linear Models) implemented in the statistical package SAS (version 6.04; SAS Institute, Inc, Cary, NC). A specialized t test (LSD, an option in the GLM procedure of SAS), which controls for the comparison-wise error, was employed for subsequent tests of statistical significance. Correlation coefficients were computed using the software package KALEIDAGRAPH, version 2.0 (Synergy Software, Reading, PA).

Results

Growth cone behavior is undoubtedly derived from the composite activities of many separate underlying processes. This poses significant experimental challenges in relating calcium changes to changes in growth cone behavior. Some of these processes may well be modified by intracellular calcium signals, while others may be independent of this second messenger system. The present study was designed to minimize the experimentally confounding effects of this complexity by focusing on a quantitative description of one discrete component of growth cone behavior, the behavior of the actin-based filopodia. While neurite elongation in *Helisoma* neurons B5 is very slow (ranging from 10 to 20 μm/hr), filopodial behavior changes on a time scale comparable to the changes in $[Ca^{2+}]_i$ experimentally evoked. In the following experiments, $[Ca^{2+}]_i$ was perturbed and simultaneously measured with the calcium indicator fura-2. To avoid

any potentially interacting calcium-buffering effects of fura-2, the morphology of filopodia was assessed in an identical experiment on unloaded growth cones and then directly correlated with the observed changes in $[Ca^{2+}]_i$. This focus on a single component of growth cone behavior undoubtedly accounts for the tight correlation that was ultimately found with altering calcium levels.

Experimental modification of filopodial length and number

In the first series of experiments, the extracellular potassium concentration ($[K^+]_o$) was raised 10-fold (from the normal concentration of 1.7 mM to 17 mM) with the logic that elevation of $[K^+]_o$ would depolarize neurons and, in turn, open voltage-dependent calcium channels (see next section). Growth cone morphology underwent a stepwise series of distinct changes following this treatment. In a first phase of response, lasting from 2 to 10 min, some filopodia gradually elongated two- to threefold (Fig. 1). The second phase, partially overlapping with the first phase, was characterized by a massive loss of filopodia. Filopodial loss was readily reversible by returning to normal medium (1.7 mM KCl), after which filopodia quickly reappeared. The experiments below partition these two phases of filopodial
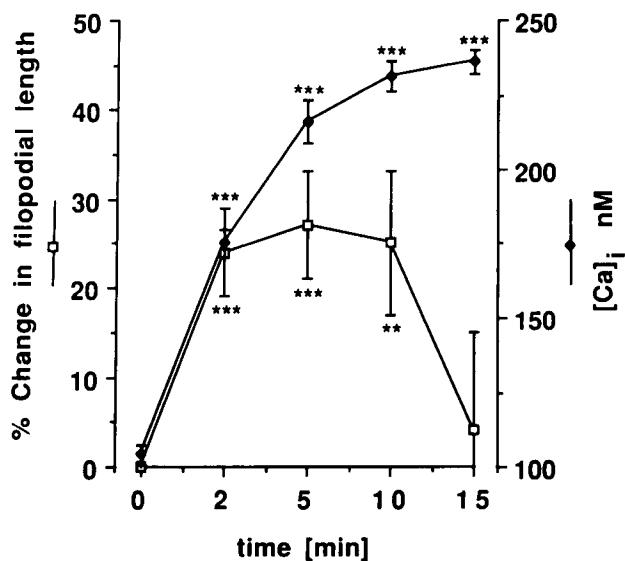
Figure 2. Short-term effects of a 10-fold elevation of extracellar [K$^+$] on estimated levels of [Ca^{2+}]$_i$ and growth cone filopodial *length*. Elevation of [K$^+$]$_o$ resulted in a rise in [Ca^{2+}]$_i$ from pretreatment levels of 104 ± 3 nM ($n = 48$–54 growth cones of nine neurons) to peak values of 236 ± 5 nM within 15 min ($p < 0.001$). Filopodia displayed a marked, but transient, elongation from pretreatment levels (normalized, 0%) for ~10 min (by 27% at 5 min; 21–26 growth cones, $p < 0.001$) in response to a 10-fold rise in [K$^+$]$_o$. After this elongation, filopodial length returned to pretreatment levels. Note the short time course of elongation in comparison to the longer-lasting effect on filopodial loss shown in Figure 3*b*. Significance levels: **, $p < 0.01$; ***, $p < 0.001$ compared to pretreatment levels.

response and directly assess the effect of elevated [K$^+$]$_o$ on measured levels of intracellular calcium.

A rise in [Ca^{2+}]$_i$ precedes filopodial elongation

We obtained fura-2 measurements of [Ca^{2+}]$_i$ in response to a 10-fold increase in [K$^+$]$_o$: [Ca^{2+}]$_i$ slowly increased from basal levels of 104 ± 3 nM to reach 175 ± 5 nM ($n = 48$–54 growth cones from nine neurons; $p < 0.001$) after 2 min and peak levels of 236 ± 5 nM after 15 min (Fig. 2). These changes in [Ca^{2+}]$_i$ were tightly correlated in time with the initial morphological effect of raised KCl, the elongation of filopodia. The mean filopodial length was significantly increased by 2 min after the start of the KCl exposure, reaching a maximum extension of 27 ± 6% ($n = 21$–26, $p < 0.001$ compared to pretreatment) at 5 min (Fig. 2). The effect on elongation was over after 15 min, though [Ca^{2+}]$_i$ was still at its maximum. There is an apparent overlap with the second phase of the response, the massive shortening and loss of filopodia, with this initial elongation. This is evident from the fact that net elongation is rather small (27%) even though individual filopodia elongate by as much as 200–300%. On a given growth cone, one observes concomitant elongation of some and shortening of other filopodia.

To test whether the transient effect on filopodial elongation was dependent on calcium influx, the preceding experiment was repeated in calcium-free medium containing 17 mM KCl, thereby allowing depolarization but preventing the influx of calcium. This treatment produced no significant rise in [Ca^{2+}]$_i$ ($n = 12$–25 growth cones of four neurons) within 45 min. Moreover, filopodial length remained unchanged for the initial 10 min, in which filopodial elongation had taken place in the previous experiment. There was, however, a significant decrease in filo-

podial length at 15 min ($n = 23$, $p = 0.004$). This reduction in filopodial length could be a result of the lack of extracellular calcium in the medium, a treatment known to transiently decrease [Ca^{2+}]$_i$ by 16% (Rehder et al., 1991). We therefore next tested whether the exposure to a calcium-free medium alone would have an effect on filopodial length. This treatment also failed to trigger filopodial elongation but led to a decrease in length at 10 min and 15 min ($n = 14$, $p = 0.006$ at 15 min) compared to pretreatment length; the values at both time points, however, were not significantly different from the conditions in calcium-free medium plus 17 mM KCl. The decrease seen in calcium-free medium plus 17 mM KCl, therefore, can be attributed to the calcium-free condition alone. These results suggest that the initial effect seen on elongation of filopodial length is a depolarization-evoked, calcium-dependent phenomenon.

A rise in [Ca]$_i$ precedes filopodial loss

The second phase of behavioral change evoked by raising [K$^+$]$_o$ was the loss of filopodia (Fig. 1). This effect, however, although initially overlapping with filopodial elongation, was longer lasting and required observation periods up to 2 hr. After a 10-fold increase in extracellular potassium, [Ca^{2+}]$_i$ increased from basal levels to reach peak levels after 15 min, as previously described. From this time on, [Ca^{2+}]$_i$ decreased to establish a new plateau value of around 180 nM for the remainder of the 2 hr observation period (Fig. 3*a*). The [Ca^{2+}]$_i$ remained elevated throughout the KCl exposure but could be readily reversed by returning to normal (1.7 mM KCl-containing) medium. For instance, within 10 min of restoration to normal [K$^+$]$_o$, the [Ca^{2+}]$_i$ had returned to 108 ± 4 nM, a value not significantly different from pretreatment levels.

The potassium-evoked rise in [Ca^{2+}]$_i$ was closely paralleled in time by a loss of filopodia. Filopodial loss (expressed as percentage loss from pretreatment levels) reached 13 ± 3% at 5 min ($n = 52$–64 growth cones on nine neurons, $p < 0.001$) and 35 ± 4% at 15 min, when the [Ca^{2+}]$_i$ reached its maximum (Fig. 3*b*). Filopodial loss was maximal at 53 ± 4% after 30 min, and thereafter the filopodial number remained reduced for another 30 min. It is interesting to note that the loss of filopodia followed the rise in [Ca^{2+}]$_i$ with a short latency. Growth cones continued to grow in 17 mM KCl medium, and naturally occurring branching and fasciculation with other neurites made observations of individual growth cones impossible in the second hour of the observation period. Consequently, growth cone activity could not be quantified for this time interval. After 2 hr, however, growth cones on these neurons looked qualitatively indistinguishable from untreated growth cones. They exhibited a fully developed veil and numerous filopodia despite the continuously elevated levels of [Ca^{2+}]$_i$, suggesting full filopodial recovery. Experiments described below confirmed this impression: recovery of filopodia took place in 34 mM KCl containing medium, where growth was much slower and branching occurred less frequently so that identified growth cones could be observed for the entire duration of the experiment (see below).

Calcium influx is the main source of the elevated [Ca^{2+}]$_i$ observed. Repeating the experiment in calcium-free medium containing 17 mM KCl produced no significant rise in [Ca^{2+}]$_i$ ($n = 12$–25 growth cones of four neurons; Fig. 3*a*). Moreover, filopodial numbers on 35–40 growth cones of four neurons were also not significantly affected by this treatment (Fig. 3*b*). Moreover, growth cones ($n = 30$ growth cones of five neurons) displayed no significant change in filopodial number in calcium-
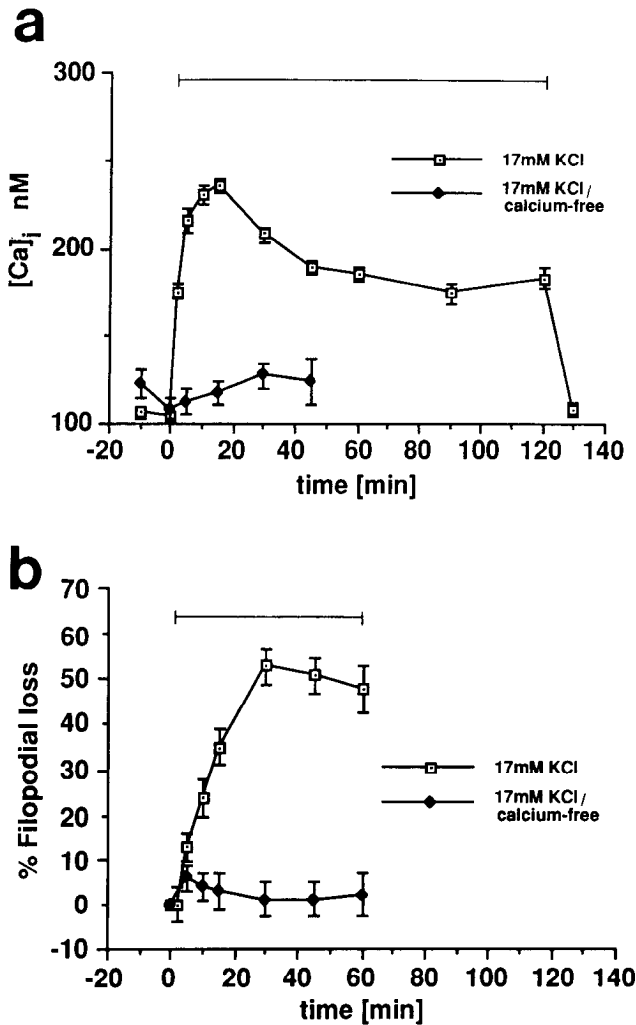
Figure 3. Longer-term effects of a 10-fold elevation of extracellular [K$^+$] on estimated levels of [Ca^{2+}]$_i$ and on filopodial *number.* The *bar* indicates the presence of elevated [K$^+$]$_o$. *a,* [Ca^{2+}]$_i$ rose to peak values within 15 min and thereafter decreased to plateau values of around 180 nM. This level was maintained as long as the [K$^+$]$_o$ remained elevated, but could be rapidly reversed after washout. Raising the [K$^+$]$_o$ in calcium-free medium did not result in a significant rise in [Ca^{2+}]$_i$ (n = 12–25 growth cones of four neurons). *b,* Filopodial loss follows the rise in [Ca^{2+}]$_i$ after a short latency. After 15 min, filopodial loss reached 35 ± 4% (52–64 growth cones of nine neurons, p < 0.001), and maximal filopodial loss was reached at 30 min (53 ± 4%). Filopodial numbers could not be assessed in the second hour of the experiment, because continued growth (branching and fasciculation of growth cones with other neurites) made it impossible to track identified growth cones. Raising the [K$^+$]$_o$ in calcium-free medium had no effect on filopodial number (n = 35–40 growth cones of four neurons).

free medium alone. We therefore conclude that the effect on filopodial loss, as was previously concluded for filopodial elongation, results from a depolarization-induced increase in calcium influx.

Dose dependency of calcium's action on filopodial behavior

In order to examine more closely the correlation between [Ca^{2+}]$_i$ and filopodial morphology, we next attempted to generate both smaller or larger rises in [Ca^{2+}]$_i$ to examine the dose dependency of calcium's action on filopodial behavior. After a small elevation of [K$^+$]$_o$ (5- instead of 10-fold), [Ca^{2+}]$_i$ rose from pretreatment levels of 104 ± 3 nM (33 growth cones of five neurons)
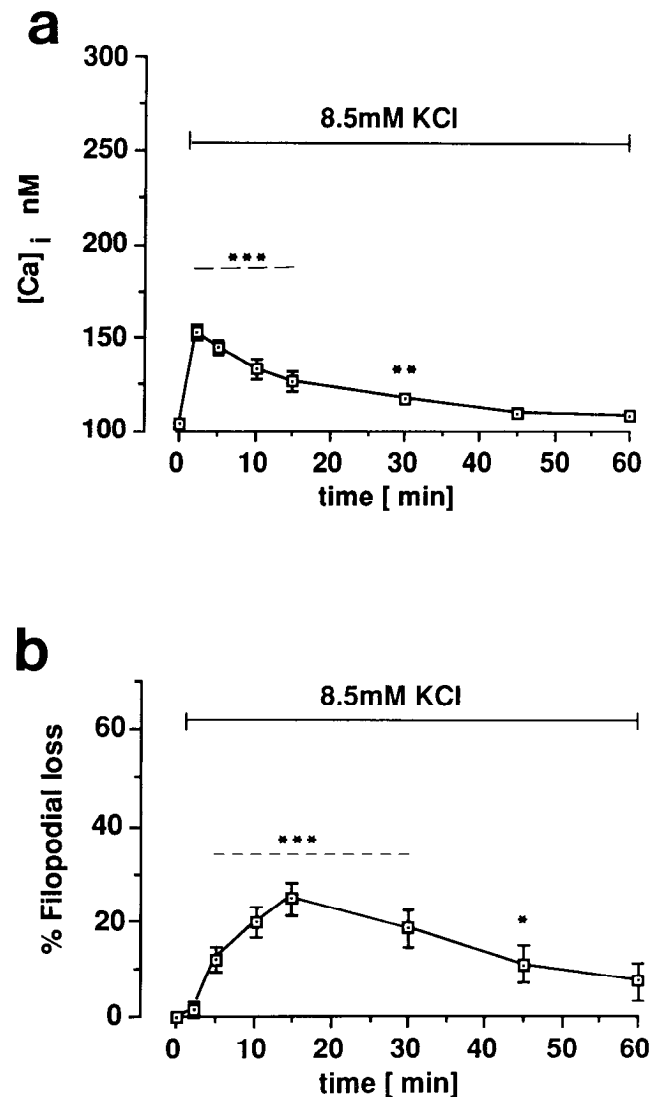

Figure 4. Effect of a fivefold elevation of [K$^+$]$_o$ on estimated levels of [Ca^{2+}]$_i$ and on filopodial number. *a,* Upon the change from normal medium to 8.5 mM KCl, [Ca^{2+}]$_i$ rose from pretreatment levels of 104 ± 3 (n = 33 growth cones of five neurons) to reach its maximum of 152 ± 4 nM (p < 0.001) at 2 min. Thereafter, [Ca^{2+}]$_i$ fully recovered within 45 min. *b,* Corresponding to the smaller rise in [Ca^{2+}]$_i$ (as compared to the 10-fold elevation of [K$^+$]$_o$ in Fig. 3), there was a small filopodial loss that reached 25 ± 4% (n = 47 growth cones of five neurons) and fully recovered within 60 min. Significance levels: *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared to pretreatment.

to 152 ± 4 nM by 2 min (p < 0.001), and from thereon decreased to recover fully after 45 min (Fig. 4a). This small rise in [Ca^{2+}]$_i$ of about 50 nM caused only a small effect on filopodia. Filopodial elongation (phase 1) was statistically significant, however, from controls at the 5 min time point, when it reached 9 ± 4% (n = 17 growth cones of six neurons, p < 0.05). Filopodial number (phase 2), however, was reduced by 12 ± 3% (n = 47 growth cones of five neurons, p < 0.001) at 5 min and exhibited its strongest effect of 25 ± 4% at 15 min (Fig. 4b). Thereafter, filopodial loss was reversed and recovery was complete at 60 min. A smaller depolarization, therefore, had only a transient effect on [Ca^{2+}]$_i$ and both filopodial length and number.

Raising [K$^+$]$_o$ 20-fold (from 1.7 to 34 mM) led to a greater rise in [Ca^{2+}]$_i$ and had an increased effect on the filopodia. [Ca^{2+}]$_i$

a

34mM KCl

[Ca]$_i$ nM

400

300

200

100

0   20   40   60   80   100   120   140

time [min]

b

34mM KCl

% Filopodial loss

70

50

30

10

-10

0   20   40   60   80   100   120   140
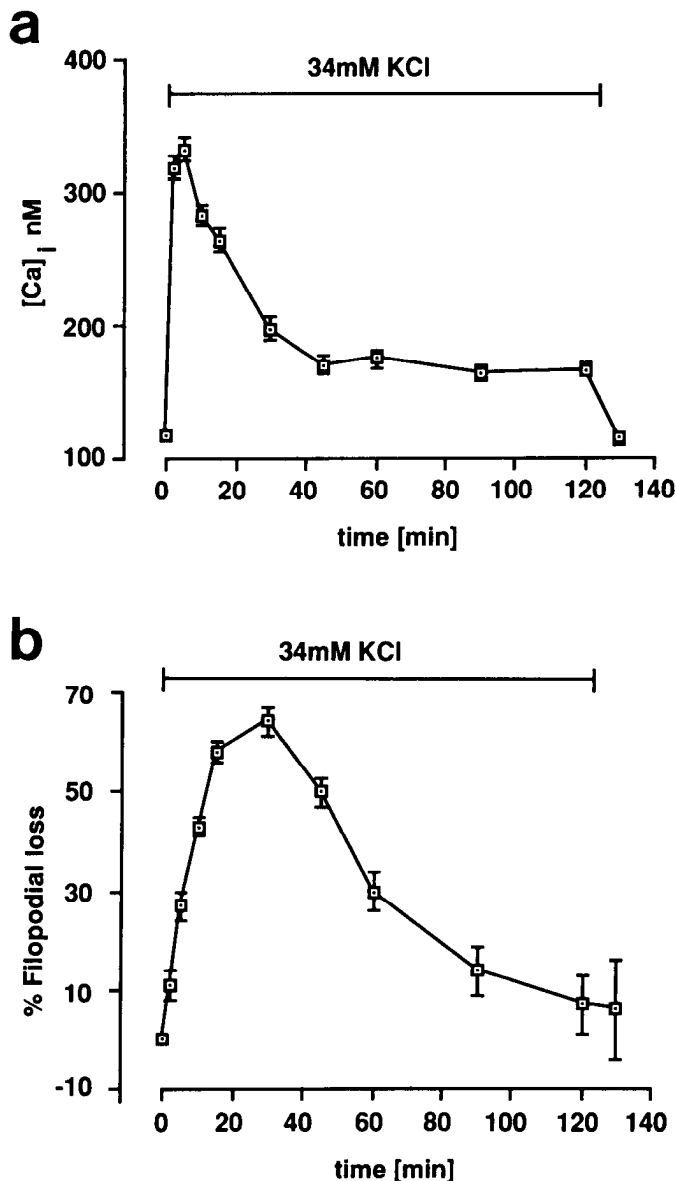
time [min]

Figure 5. Effect of a 20-fold elevation of [K$^+$]$_o$ (from 1.7 to 34 mM) on estimated levels of [Ca^{2+}]$_i$ and on filopodial number. *a*, This elevation of [K$^+$]$_o$ resulted in [Ca^{2+}]$_i$ rising more quickly than seen previously and also reached higher values (334 ± 9 nM; *n* = 25–51 growth cones of seven neurons, *p* < 0.001). From peak levels, [Ca^{2+}]$_i$ decreased to a plateau of around 180 nM. *b*, Filopodial loss was more pronounced under these conditions, compared to previous treatments. At 5 min, filopodial loss had already reached 27 ± 3% (*n* = 43–71 growth cones of six neurons, *p* < 0.001) and maximal filopodial loss was 64 ± 3% at 30 min. Interestingly, filopodial numbers thereafter recovered to pretreatment levels within 120 min, despite the fact that [Ca^{2+}]$_i$ was still significantly elevated at a plateau of around 180 nM.

rapidly rose from basal levels of 117 ± 3 nM to 320 ± 9 nM at 2 min (*n* = 25–51 growth cones of seven neurons, *p* < 0.001) and reached peak levels of 334 ± 9 nM at 5 min. Thereafter, [Ca^{2+}]$_i$ gradually decreased to a plateau of about 180 nM, which was maintained as long as the [K$^+$]$_o$ remained elevated (Fig. 5*a*). Filopodial elongation (phase 1) was observed throughout the first 10 min, as was the case in 17 mM KCl medium, and reached a maximum of 41 ± 11% over controls (*n* = 21 growth cones of five neurons, *p* = 0.001). Filopodial loss (phase 2), in turn, reached 11 ± 3% after 2 min (*n* = 43–71 growth cones from

six neurons, *p* < 0.001), 43 ± 2% after 10 min, and increased to reach its maximum of 64 ± 3% at 30 min (Fig. 5*b*). From this time, filopodial number steadily recovered and reached pretreatment levels after 120 min in the continuous presence of elevated [K$^+$]$_o$. Filopodial recovery took place while the [Ca^{2+}]$_i$ was still elevated. Thus, for pragmatic purposes, one might consider that in addition to the two effects on filopodial behavior demonstrated so far, there exists a third phase in response to the evoked rise in [Ca^{2+}]$_i$, namely, filopodial "adaptation" to sustained elevations of [Ca^{2+}]$_i$.

A second perturbation that did not depend on the activation of voltage-gated calcium channels was also employed to increase [Ca^{2+}]$_i$ in growth cones. The calcium ionophore 4-bromo A23187 (0.75 μM) was recently shown to raise [Ca^{2+}]$_i$ in growth cones in this system (Rehder et al., 1991). The effect on filopodial number is shown in micrographs in Figure 6*a* and quantitatively expressed in Figure 7*b*. After application of ionophore, [Ca^{2+}]$_i$ was elevated from rest levels of 135 ± 2 nM to 459 ± 36 nM (57 growth cones from seven neurons, *p* < 0.001) within 5 min, whereupon [Ca^{2+}]$_i$ decreased to a plateau around 260 nM (Rehder et al., 1991; Fig. 7*a*). Filopodial elongation (phase 1) was prominent between 5 and 30 min and reached maximal levels of 45 ± 11% after 15 min (*n* = 15 growth cones of four neurons). Five minutes after application of the ionophore, the number of filopodia (phase 2) was dramatically reduced by 53 ± 3% of pretreatment levels (*n* = 46 growth cones of six neurons, *p* < 0.001), reaching its maximal loss of 72 ± 4% at 15 min (Fig. 7*b*). However, despite the continuous presence of the ionophore, the number of filopodia was restored to pretreatment levels within 60 min, indicating filopodial "adaptation" (phase 3) to sustained elevations of [Ca^{2+}]$_i$, as was the case with 34 mM KCl.

A small increase of about 30 nM in [Ca^{2+}]$_i$ can be produced by the application of 0.75 μM 4-bromo A23187 in a calcium-free medium (Fig. 7*a*). This rise is likely due to ionophore-mediated release from internal stores of calcium, since influx is not possible under this condition (Rehder et al., 1991). Employing this treatment, the filopodial number (phase 2) was reduced by 15 ± 4% of controls within 10 min (*n* = 16 growth cones of four neurons, *p* = 0.005) and thereafter recovered (Fig. 7*b*). Filopodial length (phase 1) was not assessed in this experiment. This experiment demonstrates that an increase in [Ca^{2+}]$_i$ of as small as 30 nM can significantly alter filopodial behavior.

Isolated growth cones

An earlier study demonstrated that growth cones isolated from the remainder of the neuron by neurite transection are capable of controlling their [Ca^{2+}]$_i$ to a similar degree as growth cones attached to their parent neurons. With this preparation, we determined whether the treatments described above acted directly on the growth cone, or indirectly through the cell body to alter filopodial behavior. Upon transection, [Ca^{2+}]$_i$ in the isolated growth cone is transiently raised to values exceeding 1000 nM and quickly restored toward basal levels within 10 min (Rehder et al., 1991). Interestingly, this rise in [Ca^{2+}]$_i$, albeit quite transient, was also accompanied by a significant elongation of filopodia that rapidly reached a maximal value of 33 ± 5% at 5 min (*n* = 19–23, *p* < 0.001). The effect on elongation lasted for 10 min.

After 60 min, isolated growth cones appeared normal, with fully developed veils and filopodia (Fig. 6*b*). Levels of intracellular calcium were also restored to basal values at this time after transection (Rehder et al., 1991). Isolated growth cones,
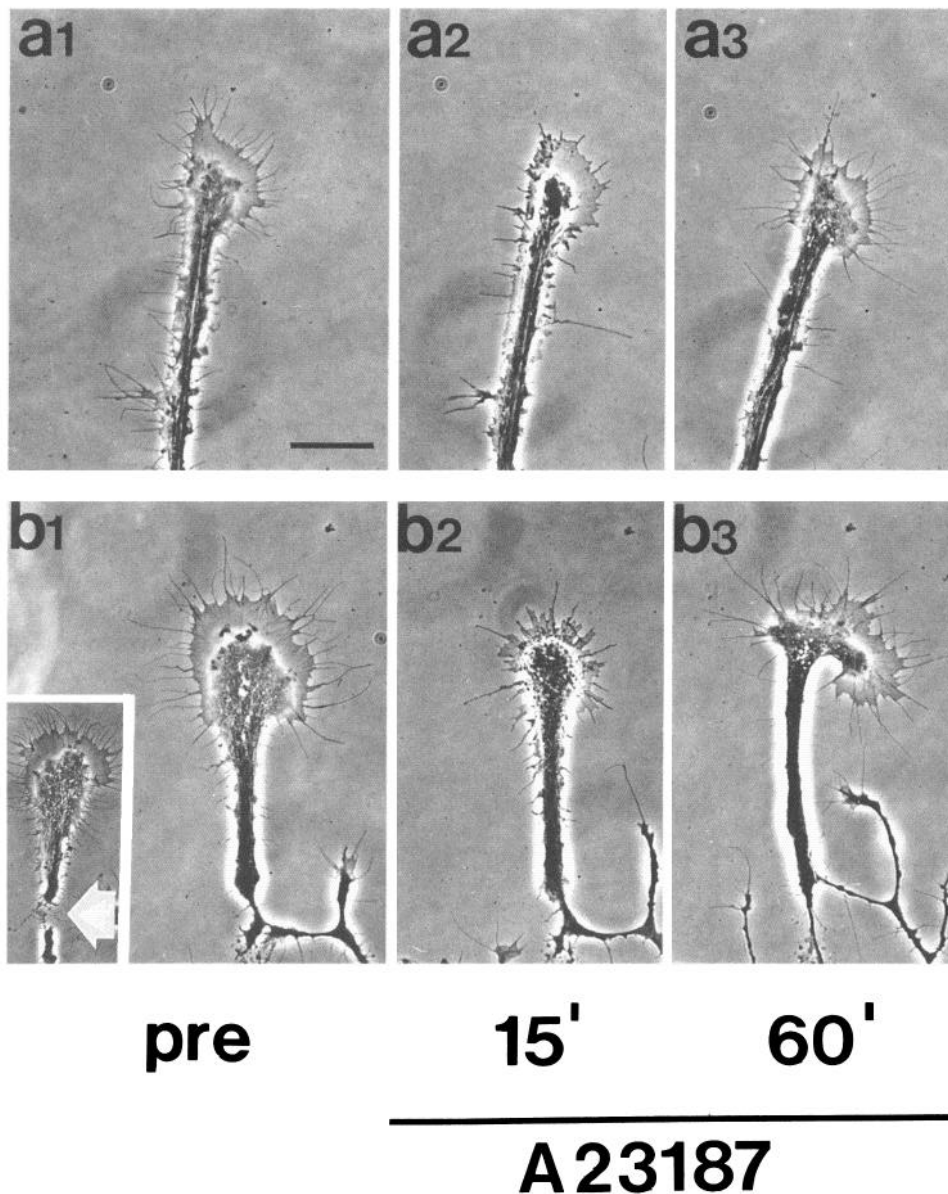
Figure 6. Phase-contrast micrographs demonstrating the effects of the calcium ionophore 4-bromo A23187 (0.75 μM) on filopodial numbers in attached (a) and isolated (b) growth cones. Note the decrease and subsequent restoration of filopodia before (a_1, b_1), at 15 min (a_2, b_2), and at 60 min after ionophore treatment (a_3, b_3). The *inset* in b_1 shows the growth cone demonstrated in b_1 55 min prior to the experiment, that is, 5 min after isolation (*arrow* marks transection site). At later time points (b_1–b_3), other growth cones of this neuron contacted the isolated growth cone and partly obscured the fact that it had been previously isolated. Scale bar, 20 μm.

in addition to their ability to display filopodial elongation, also showed filopodial loss upon raising the $[K^+]_o$. After a 10-fold increase in $[K^+]_o$, filopodial loss reached $22 \pm 6\%$ ($n = 39$–43 growth cones of nine neurons) after 10 min, and maximal levels of $79 \pm 6\%$ at 45 min (Fig. 8b). With the same treatment, $[Ca^{2+}]_i$ rose from pretreatment levels of 136 ± 4 nM to 274 ± 9 nM ($n = 37$–43 growth cones from nine neurons, $p < 0.001$) after 5 min (Fig. 8a). From peak values, $[Ca^{2+}]_i$ decreased to plateau values of around 180 nM, as was the case in intact growth cones. As might be expected, essentially identical results were obtained with the ionophore 4-bromo A23187 (0.75 μM) on isolated growth cones. This treatment has previously been shown to evoke a substantial rise in $[Ca^{2+}]_i$ that was very similar to the one seen in attached growth cones (Rehder et al., 1991). Accordingly, filopodial loss reached $20 \pm 6\%$ of controls in 5 min, and reached its highest level after 30 min with $63 \pm 6\%$ ($n = 23$ growth cones from six neurons). Filopodia were then restored between 30–60 min and reached pretreatment levels after 60 min (Fig. 6b), despite elevated levels of intracellular calcium. Thus, iso-

lated growth cones were also able to display the third phase of filopodial behavior previously described for attached growth cones, namely, filopodial adaptation. Therefore, local rises of $[Ca^{2+}]_i$ in the growth cone proper, independent of the treatment used to evoke these rises, can control filopodial behavior.

Correlation between $[Ca^{2+}]_i$ and filopodial behavior

The data provided by the various experiments of this investigation allow one to analyze statistically the relationship between a rise in $[Ca^{2+}]_i$ and filopodial length and number. The peak values of $[Ca^{2+}]_i$ reached during the treatments previously described were plotted against the maximal effect observed on filopodial length (Fig. 9a) and filopodial loss (Fig. 9b), thus bringing together the two different types of data from the preceding experiments. With the expectation of a physiological dose–response curve, we approximated the data by a logarithmic function of the form $y = a + b \cdot \log x$. Correlation coefficients of $r = 0.99$ and $r = 0.98$ were found for calcium and filopodial length and loss, respectively, indeed, suggesting a strong cor-
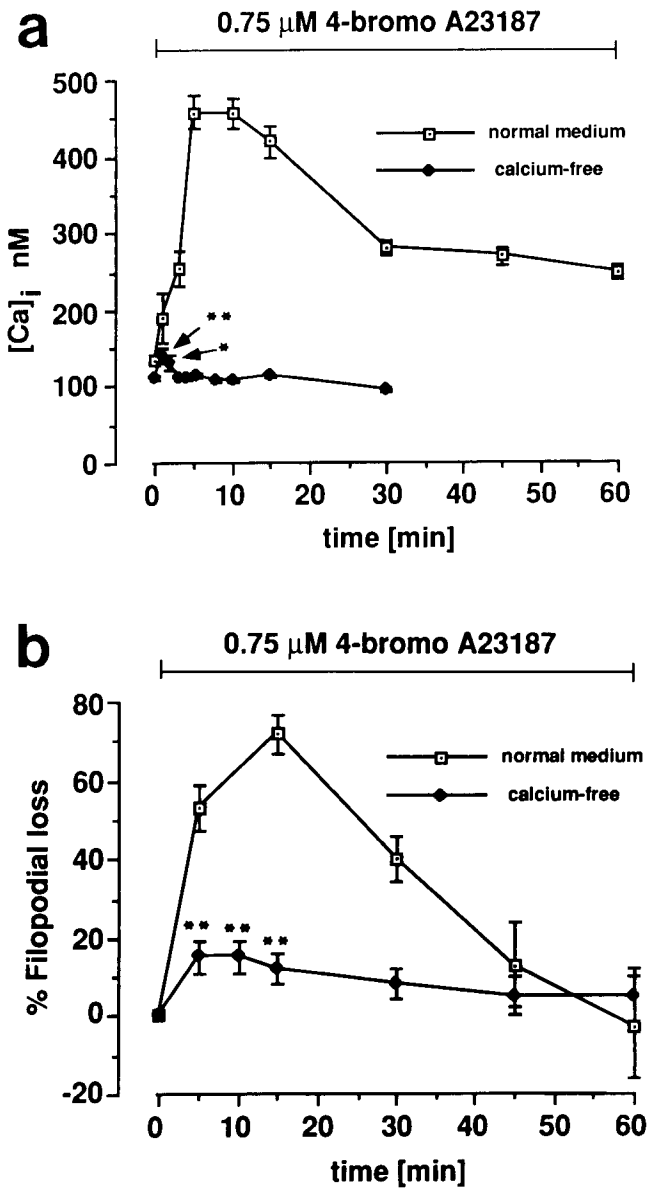
a 0.75 µM 4-bromo A23187

a 17mM KCl

b 0.75 µM 4-bromo A23187

b 17mM KCl

Figure 7. Effect of calcium ionophore 4-bromo A23187 on estimated levels of $[Ca^{2+}]_i$ and on filopodial numbers. *a,* Upon ionophore addition (0.75 µM), $[Ca^{2+}]_i$ rose from pretreatment levels to 459 ± 36 nM within 5 min (*n* = 57 growth cones of seven neurons) but was restored to a new plateau level of around 260 nM after 30 min. After addition of the same concentration of ionophore in calcium-free medium, however, $[Ca^{2+}]_i$ only rose from rest levels of 110 ± 3 nM (*n* = 12 growth cones of two neurons) to 141 ± 10 nM within the first minute (*p* = 0.007) and thereafter quickly recovered to pretreatment values (data taken from Rehder et al., 1991). *b,* Filopodial numbers were reduced to 53 ± 3% (*n* = 46 growth cones of six neurons, *p* < 0.001) by 5 min, and the maximal loss occurred at 15 min (72 ± 4%). The filopodia fully recovered within 60 min, despite the elevated values of $[Ca^{2+}]_i$. Addition of the ionophore in calcium-free medium caused a maximal filopodial loss of 15 ± 4% at 5 min (*n* = 16 growth cones of four neurons). Thereafter, filopodial numbers increased again and were not different from pretreatment levels at 45 min. Significance levels: *, *p* < 0.05; **, *p* < 0.01 compared to pretreatment.

relation between the $[Ca^{2+}]_i$ and both parameters of filopodial behavior. The fact that both data sets (KCl and 4-bromo A23187) fit the curve well suggests that it is indeed the rise in $[Ca^{2+}]_i$ that brings about the effects seen, and that it does not matter by which method this rise is induced. Since the number of filopodia
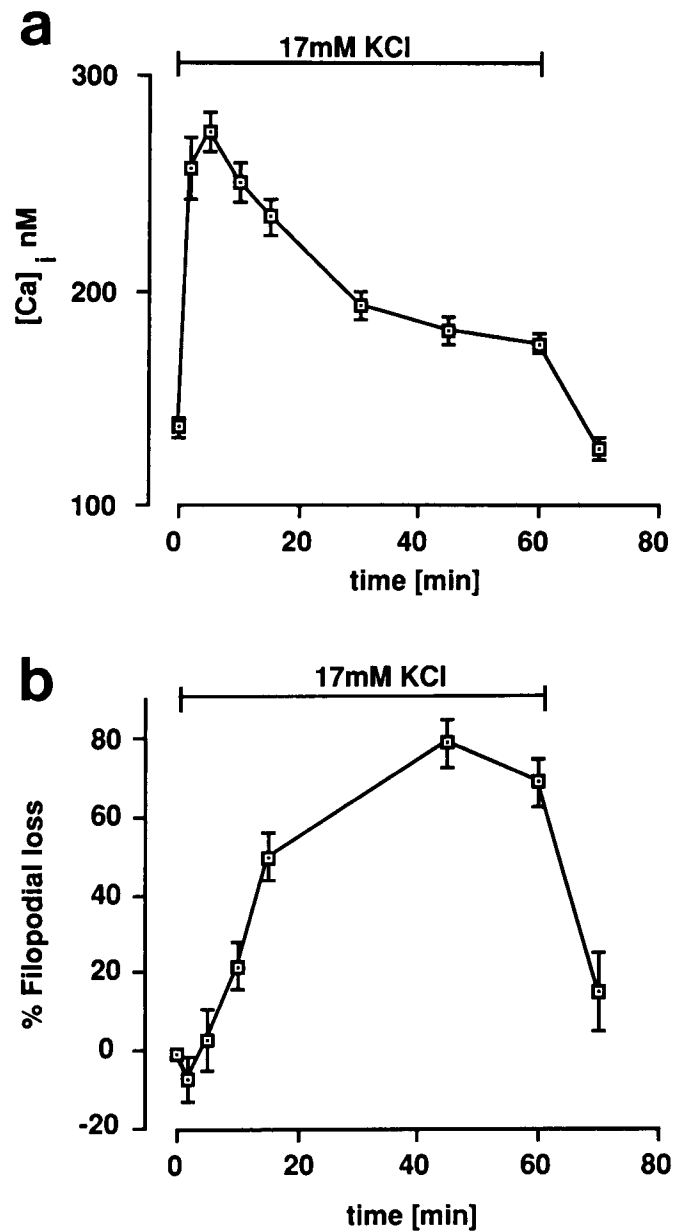
Figure 8. The response of *isolated* growth cones to a 10-fold elevation of $[K^+]_o$ on estimated levels of $[Ca^{2+}]_i$ and on filopodial number. *a,* Upon the medium change, $[Ca^{2+}]_i$ rose to peak levels of 274 ± 9 nM (*n* = 37–43) at 5 min. Thereafter, $[Ca^{2+}]_i$ steadily decreased to plateau levels of around 180 nM, as was the case in attached growth cones. Upon returning to normal medium, $[Ca^{2+}]_i$ was reduced to pretreatment levels within 10 min. *b,* Filopodial loss gradually increased and reached maximum levels of 79 ± 6% at 45 min (*n* = 39–43 growth cones). Upon returning to normal, medium filopodia were also quickly restored.

returned to pretreatment values after extended treatment periods, a comparison of plateau levels of $[Ca^{2+}]_i$ and filopodial loss showed no correlation (correlation coefficient *r* = 0.2).

Discussion

Filopodia on neuronal growth cones are thought to be important structures for directing neuronal outgrowth both *in vitro* (Letourneau, 1979; Bray and Chapman, 1985; Hammarback and Letourneau, 1986; Goldberg and Burmeister, 1989) and *in vivo* (Bentley and Keshishian, 1982; Taghert et al., 1982; Bentley and Toroian-Raymond, 1986; O'Connor et al., 1990). Time-
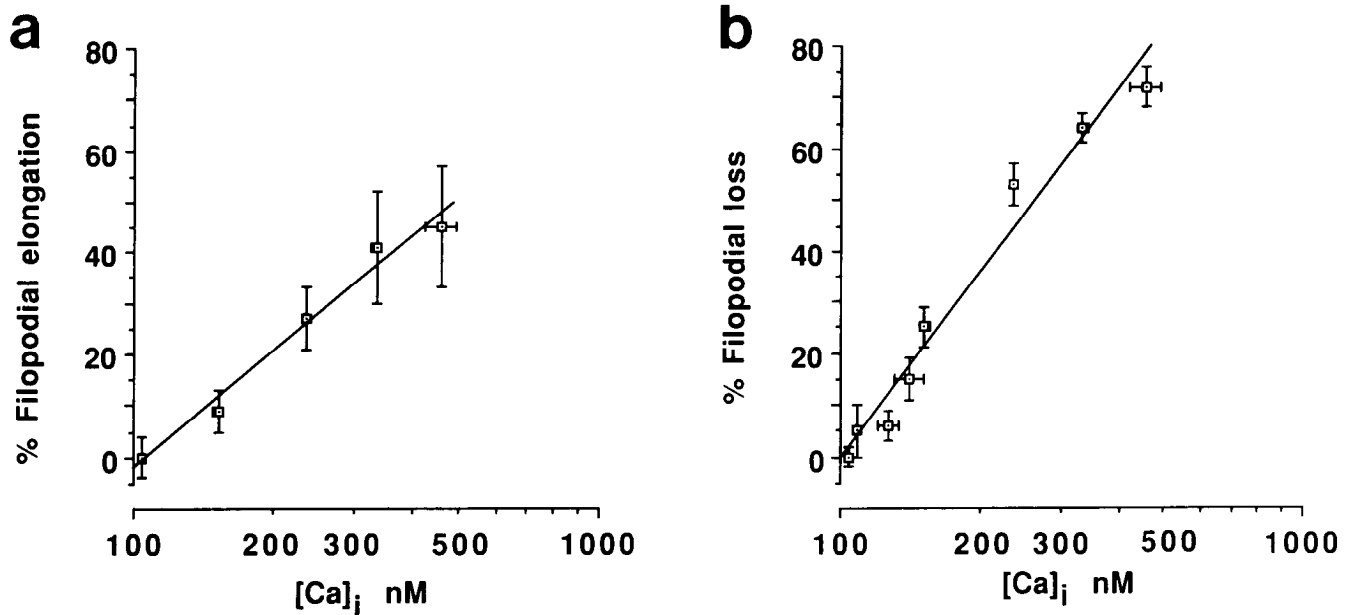
Figure 9. Statistical correlation between estimated $[Ca^{2+}]_i$ and filopodial length (*a*) and filopodial loss (*b*) in attached growth cones. Peak values of $[Ca^{2+}]_i$ reached during each treatment (for different $[K^+]_o$ and ionophore treatments) were plotted against maximal values of filopodial length (correlation coefficient $r = 0.99$) and filopodial loss ($r = 0.98$), indicating a strong correlation between $[Ca^{2+}]_i$ and both phases of filopodial behavior. Where error bars are not visible, they are confined in the data point.

lapse studies from a variety of preparations have revealed that filopodia are highly dynamic structures that protrude from the body of the growth cone in a fanlike fashion. Changes in filopodial patterns depend on the molecular terrain the growth cone traverses *in vivo* (Roberts and Taylor, 1983; Taylor and Roberts, 1983; Tosney and Landmesser, 1985; Caudy and Bentley, 1986), with growth cones exhibiting a more complex morphology often with elongated filopodia at "decision points" encountered along its route (Taghert et al., 1982; Raper et al., 1983; Tosney and Landmesser, 1985; Eisen et al., 1986; Holt, 1989).

Possible significance of filopodial regulation

Although it is generally accepted that filopodia could play a role in the searching behavior of growth cones, it is worth examining what the advantages of subcellular regulation of filopodial behavior might be. Given the potentially complex nature of the molecular terrain over which neuronal growth cones must traverse, a variety of qualitatively and quantitatively different cues may be encountered, some even simultaneously. It is possible to envision the growth cone traversing terrain that periodically signals the growth cone to alter its "searching behavior" by increasing or decreasing the length and/or number of filopodia. Elongation of filopodia would effectively increase the specific area "surveyed" by the growth cone and potentially affect neuronal guidance. Additionally, rapid elongation would allow a filopodium to bridge distances within minutes, which otherwise would be hours away assuming standard rates of growth cone advance. Alternatively, it is possible to envision the molecular terrain signaling the growth cone to decrease its "searching behavior." In this scenario, one might envision a growth cone elongating in the absence of filopodia as has been shown after application of cytochalasin (Argiro et al., 1984; Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986; Letourneau et al., 1987; McCaig, 1989). Thus, the searching behavior of the growth cone would be decreased and it would be biased

toward continuing along an established path, despite potential cues in the surrounding environment. Growth cones of neurons following established tracts or fascicles have indeed been described as being less morphologically complex compared to pioneer neurons (Lopresti et al., 1973; Taylor and Roberts, 1983; Caudy and Bentley, 1986).

An active control of the number and length of filopodia would serve, therefore, an important role in providing the growth cone with an optimal flow of information about its environment. Given the time course of filopodial elongation, a variety of different scenarios is possible. The elongating filopodium could contact an important guiding structure and then be transformed into the future neurite by dilation, thereby laying down the direction of future growth as described for pioneer neurons of the grasshopper (O'Connor et al., 1990). Alternatively, a filopodium could come in contact with its final target and the growth cone could be transformed into a presynaptic structure (Gottlieb and Glaser, 1980; Tsui et al., 1985). In a third scenario, filopodia may not reach a significant signal at all and would subsequently be retracted. Depending on the signal and the way it is translated by the growth cone, environmental cues could then activate the appropriate building blocks of growth cone behavior, such as turning toward or away from a cue, increasing the growth rate or stopping. Taken together, the ability to regulate physiologically the number and length of filopodia on a growth cone, could have great significance for the final processes of neuronal pathfinding and synaptogenesis.

"Behavioral" correlates of changes in $[Ca^{2+}]_i$

A large literature exists on the effects of both intra- and extracellular calcium concentrations on growth and motility in neurons (Anglister et al., 1982; Suarez-Isla et al., 1984; Cohan et al., 1987; Mattson and Kater, 1987; Kater et al., 1988; Mattson et al., 1988c; Campenot and Draker, 1989; Robson and Burgoyne, 1989; Silver et al., 1989; Fields et al., 1990a,b; Kater
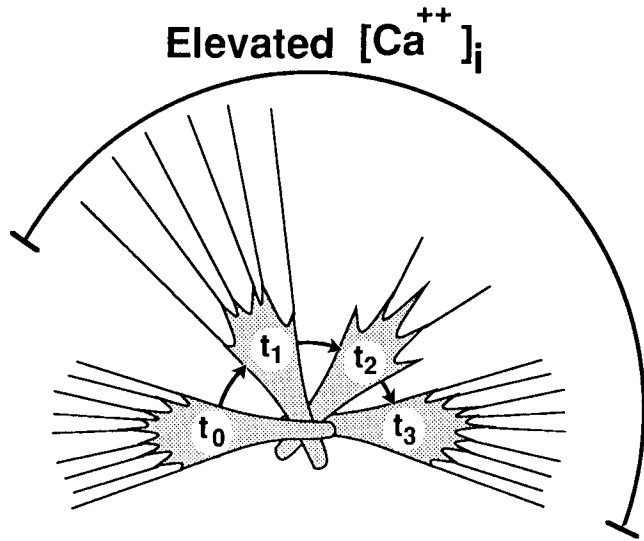
Elevated [Ca^{++}]$_i$

Figure 10. Schematic diagram of three different phases of filopodial behavior sequentially elicited by a constant duration stimulus that evokes an increase in [Ca^{2+}]$_i$. At basal levels of [Ca^{2+}]$_i$, growth cones exhibit numerous filopodia (t_o). A rise in [Ca^{2+}]$_i$ results in a stereotyped series of the following filopodial activities: filopodial elongation (t_1), filopodial loss (t_2), and filopodial adaptation (t_3). The reduction in filopodial number indicated in t_1 reflects the fact that filopodial loss partially overlaps with filopodial elongation. A potential variation in the tightly locked relationship between filopodial elongation and filopodial loss might occur in vivo, for instance, by spatially and temporally restricted environmental cues evoking only elongation or only filopodial loss, perhaps even by altering filopodial behavior only on one side of the growth cone.

and Mills, 1991; Lankford and Letourneau, 1991). Calcium is strongly implicated in regulating elements of the cytoskeleton and could, by this means, influence neuronal growth (Stossel et al., 1985; Mooseker et al., 1986; Mitchison and Kirschner, 1988; Smith, 1988; Forscher, 1989; Lankford and Letourneau, 1989, 1991; Letourneau and Shattuck, 1989; Bamburg and Bernstein, 1991).

Previous studies of neurite elongation often involved treatments that would alter [Ca^{2+}]$_i$ with the assumption that these treatments would have sustained effects on [Ca^{2+}]$_i$. However, recent studies on *Helisoma* neurons demonstrated that effects on [Ca^{2+}]$_i$ are often transient, due to the compensatory properties of calcium homeostasis (Rehder et al., 1991). Given the relatively slow nature of growth cone advance in *Helisoma* and the relatively fast rate of calcium compensatory processes, it is difficult to design investigations that will effectively test for a correlation between outgrowth and [Ca^{2+}]$_i$. The present study is not limited in the same way since the sensitive filopodial assay allows one to monitor morphological changes on a minute-by-minute time scale, corresponding to the time course of monitored changes in [Ca^{2+}]$_i$. It should be kept in mind that measurements of filopodial behavior and [Ca^{2+}]$_i$ were not performed on the same growth cones but on growth cones from parallel cultures. This approach was chosen to eliminate two problems: the introduction of a calcium buffer into a neuron, and the repeated UV illumination of growth cones. Nonetheless, the tight statistical correlation, the close temporal associations, and the control experiments in calcium-free medium clearly speak to a causal role for rises in [Ca^{2+}]$_i$ mediating these changes in filopodial behavior.

Filopodial elongation and filopodial loss

Multiple mechanisms may exist for regulating growth cone filopodia. The present investigation demonstrates that one important way to change filopodial disposition is by raising [Ca^{2+}]$_i$. Elevating the levels of this second messenger has highly predictable effects on both filopodial length and number in a sequential fashion. These results, obtained in both in attached and isolated growth cones, indicate a local control over these components of growth cone behavior. After a rise in [Ca^{2+}]$_i$, a population of filopodia first extended for approximately 10 min, followed by a massive filopodial loss (Fig. 10). The initial elongation as well as the filopodial loss were calcium dependent since depolarization with 17 mM KCl in the absence of extracellular calcium failed to produce an effect. It is, therefore, not the depolarization per se, but the rise in [Ca^{2+}]$_i$ that led to the effects on filopodia. Moreover, both events were strongly correlated with the peak values of [Ca^{2+}]$_i$ reached during each treatment. These results let us conclude that it is the transient, and not the sustained, increases in [Ca^{2+}]$_i$ (during which filopodia "adapt" as discussed below) that contain the information responsible for the initial filopodial changes. The correlation data between [Ca^{2+}]$_i$ and filopodial length and number are best fit by a logarithmic function, suggesting that relatively small increases in [Ca^{2+}]$_i$ from rest levels can effectively alter the growth cone's cytoskeleton. The smallest rise in [Ca^{2+}]$_i$ that would still significantly affect filopodial behavior was determined to be about 30 nM. This increase in [Ca^{2+}]$_i$ was produced when ionophore was administered in the absence of extracellular calcium, thereby eliminating the influx component (Rehder et al., 1991). The origin of calcium in this case is most likely from intracellular stores of calcium.

Increases in [Ca^{2+}]$_i$ could come about by a variety of stimuli ranging from electrical activity (Cohan et al., 1987) to diffusible or substrate bound environmental cues (Mattson et al., 1988b; Schmidt et al., 1991). In experiments employing *Helisoma* neurons B19 and B4, electrical stimulation, previously shown to elevate [Ca^{2+}]$_i$ (Cohan et al., 1987), has revealed a graded inhibition of filopodial number and neuronal advance (Cohan, 1990). Mattson and Kater (1987) qualitatively reported results in which they showed a decrease in filopodial number upon application of the nonbrominated A23187. Lankford and Letourneau (1989, 1991) showed evidence that calcium can act as a regulator of actin filaments in DRG growth cones. After application of 2.5 μM A23187 on DRG neurons, they showed changes, on the ultrastructural level, in the cytoskeleton, mainly a disruption or destabilization of actin filaments, but of microtubules as well. By setting [Ca^{2+}]$_i$ in calcium-permeable DRG neurons to predetermined levels, Lankford and Letourneau (1991) demonstrated that growth cone behavior is highly sensitive to changes in [Ca^{2+}]$_i$ as small as 50 nM. This value is in excellent agreement with the present findings. It is interesting to note, however, that while we find a strong decrease in the number of filopodia, Lankford and Letourneau (1989, 1991) report the main effect on actin filaments to be in the veil and not on filopodia. The difference in sensitivity between the two preparations might be explained by a difference in localization of molecules known to influence the cytoskeleton. For example, a calcium-sensitive form of α-actinin has been demonstrated in filopodia of differentiated PC-12 cells, while a calcium-insensitive form is localized in the other parts of the growth cone (Sobue and Kanda, 1989).

While KCl depolarization and ionophore treatment employed in this study acted globally on the growth cone as a whole, environmental signals could signal a growth cone to increase its "searching behavior" by selectively sending out individual filopodia in various directions by selectively and transiently raising intracellular calcium levels in discrete regions of the growth cone. The plausibility of such a local stimulus–response system is indicated by the study of local clusters of L-type calcium channels in growth cones of N1E-115 neuroblastoma cells, which can evoke local rises in $[Ca^{2+}]_i$ (Silver et al., 1990), and by the correlation observed between local filopodial elongation and local calcium influx into growth cones in *Helisoma* neurons (Davenport and Kater, 1991).

Filopodia contain an actin core and could, therefore, either be directly susceptible to an increase in $[Ca^{2+}]_i$ or could be indirectly effected by calcium via its action on actin-binding proteins (Mitchison and Kirschner, 1988; Forscher, 1989; Bamburg and Bernstein, 1991). Several proteins known to affect actin have been demonstrated immunocytochemically in neuronal growth cones and filopodia, including cross-linking agents like α-actinin (Letourneau and Shattuck, 1989; Sobue and Kanda, 1989), fimbrin (Shaw et al., 1981), and filamin (Letourneau and Shattuck, 1989), as well as membrane-anchoring proteins like integrins, talin, and vinculin (Letourneau and Shattuck, 1989). A calcium-sensitive form of α-actinin has been demonstrated in filopodia of differentiated PC-12 cells, while a calcium-insensitive form is localized in the other parts of the growth cone (Sobue and Kanda, 1989). Moreover, actin depolymerizing factor has been demonstrated to be a major component in the growth cone extending into the filopodia (Bamburg and Bernstein, 1991). Profilin, which is thought to regulate the levels of assembled actin, as well as villin and gelsolin are likely candidates involved in the regulation of the actin-based cytoskeleton. The role, however, that these proteins might play, and the way they might interact within the growth cone remain to be understood.

Filopodial adaptation

Depending on the degree of calcium influx elicited by our treatment, $[Ca^{2+}]_i$ either returned to basal levels (in the cases of 8.5 mM KCl and ionophore in calcium-free medium) or remained elevated at plateaus significantly above baseline levels (in the cases of 17 mM and 34 mM KCl, as well as 0.75 μM 4-bromo A23187). These results suggest that calcium homeostatic mechanisms intrinsic to the neuron are able to restore $[Ca^{2+}]_i$ when the generated influx is small, while a larger influx resulted in an elevated plateau. This elevated plateau indicates an equilibrium between sustained calcium influx through noninactivated calcium- or ionophore channels that cannot be completely compensated by the calcium clearance mechanisms.

The number of filopodia, however, gradually recovered despite the elevated plateau levels of $[Ca^{2+}]_i$, indicating that filopodia can "adapt" to elevated $[Ca^{2+}]_i$ and that there appears to be a wide range of $[Ca^{2+}]_i$ permissive for normal cytoskeletal organization. This finding indicates that the filopodial number is not determined by the absolute value of intracellular calcium present at any given time, since a rise from basal levels to any of these plateau values would have resulted in a considerable filopodial loss. The significant correlation between the peak values of $[Ca^{2+}]_i$ and the peak values of filopodial loss support the notion that the magnitude of filopodial loss is determined by the *magnitude of the initial calcium rise*.

Conclusion

The behavior of growth cones undoubtedly comprises many processes and control systems, each requiring independent investigation. The present study has attempted to dissect these processes by focusing on one aspect of the control of one process by a single second messenger system of the growth cone. The capacity for varying filopodial number and length by altering $[Ca^{2+}]_i$, together with the previous work on a potential sensorlike role for filopodia, reinforces the idea that these structures may act as telescoping sensory probes for guiding neuronal pathfinding.

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