Regional distribution of calcium influx into bursting neurons detected with arsenazo III

(stomatogastric ganglion/calcium channels/synaptic transmission/crustacea/optical recording)

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ABSTRACT   Absorbance changes of the metallochromic indicator arsenazo III were used in conjunction with an array of 100 photodiodes to measure changes in intracellular calcium concentration at many positions simultaneously in identified neurons of the crab stomatogastric ganglion. When stimulated with intrasomatically injected current, several of these neurons showed calcium changes all over the cell, indicating that calcium channels were distributed widely in the neuropil and on the soma. When the membrane potential was allowed to oscillate without stimulation, absorbance oscillations were detected all over the neuropil but not in the soma. A comparison between the membrane potential recorded in the soma and the calcium signal in the neuropil shows that calcium entry followed the slow voltage oscillation with the peak calcium signal detected 50–150 msec after the end of the voltage plateau.

The neurons of the stomatogastric ganglion of decapod crustaceans exhibit a complex of cell and circuit properties that underlie the generation of rhythmic motor patterns. Recordings from the soma of pyloric neurons reveal oscillations in membrane potential with attenuated spikes on the oscillation peaks (1). These oscillations are caused by a mixture of active membrane conductances (2), which cause plateau potentials (3), and by both graded and spike-evoked synaptic potentials (4).

Recordings are normally made from the cell bodies of these neurons. However, the synaptic sites are between the fine processes in the neuropil (5, 6). The plateau conductances are largely or entirely nonsomatic (2), and the spikes are initiated near the region where the axons leave the ganglion and are only passively spread through the neuropil and to the soma (7, 8). Thus, the most interesting properties of these neurons occur at a distance from the site of electrical recording (cf. refs. 9 and 10).

Voltage-dependent calcium influx is essential for both graded and spike-evoked synaptic transmission between stomatogastric neurons (4), and calcium is also thought to play a role in the generation of plateau potentials (2). Thus, information about the distribution of voltage-dependent calcium channels over the surface of these cells, and about the properties of the calcium channels at different locations, would be of great importance for understanding cell function in this system.

Two recent developments have led to a technique for approaching this problem. Calcium-sensitive molecules allow optical measurements of rapid changes in the concentration of intracellular free calcium without seriously perturbing the cells being measured (reviewed in ref. 11). Photodiode arrays allow simultaneous optical recordings from many different tissue sites (12) or from many regions of a single neuron (13–16). These techniques have been combined to examine sites of voltage-dependent calcium influx in neurons of intact ganglia (17, 18).

In this paper, we describe our use of these techniques to examine calcium transients and calcium oscillations in the neuropil of some of the cells of the stomatogastric ganglion. Calcium oscillations were first described in the soma of R15 in the abdominal ganglion of Aplysia (19, 20). These pioneering experiments used a single detector and changes in the absorbance of the metallochromic indicator dye arsenazo III to indicate changes in internal calcium concentration. In our experiments, we have used an array of photodiodes and improved light detection and electronics to increase the sensitivity, spatial resolution, and temporal resolution of the detection apparatus. With this system, we have found that calcium channels are widely distributed over the surface of many stomatogastric neurons. In addition, we have detected calcium oscillations in the neuropil, but not the soma, of some stomatogastric neurons. These oscillations are phase-shifted with respect to the soma membrane potential and are of considerable magnitude. Some of these results have been reported in abstract form (21).

MATERIALS AND METHODS

Crabs (Cancer irroratus) were obtained from the local waters of Woods Hole, MA, and were maintained in flowing natural seawater. The stomatogastric ganglion was dissected and desheathed by standard techniques (22). In some preparations, including those shown in Figs. 2 and 3, the esophageal and paired commissural ganglia were left attached to the stomatogastric ganglion to enhance the oscillations (22, 23). Ganglia were maintained and experiments were performed in saline consisting of 440 mM NaCl/11 mM KCl/13 mM $CaCl_2$/26 mM $MgCl_2$/10 mM Hepes, pH 7.6 (modified from ref. 24). In most experiments, the deisheathed ganglion was briefly exposed ($\approx$15 sec) to saline containing 1 mg of Pronase per ml (53702 Calbiochem) to facilitate electrode penetration. No difference in electrical or optical properties was noticed as a result of this procedure. Electrodes were pulled from thin-walled omega-dot glass (1011, Glass Company of America, Bargaintown, NJ). The tips were filled by capillary action with 75 mM arsenazo III (A 8891, Sigma) and then the shanks were back-filled with 3 M KCl. The arsenazo III microelectrode was used for intracellular recording and stimulation as well as for injection of dye. Neurons were filled with arsenazo III by iontophoresis into the soma. Current pulses were usually −2 to −4 nA, 500 msec duration, delivered at 1 Hz, and were given for 10–40 min. An additional 5–30 min was then allowed for further diffusion of the arsenazo III before taking data. Although data were then collected over a period of up to 2 hr, there were never more than slight changes in the

Abbreviations: VD neuron, ventricular dilator neuron; LP neuron, lateral pyloric neuron.

relative amplitudes of the absorbance signals, indicating little redistribution of arsenazo III by continued diffusion. Two suction electrodes were placed on nerves for identification of the recorded cell. Experiments were performed at room temperature (20°C–24°C in the recording chamber).

Changes in internal calcium were recorded as changes in absorbance of the dye at either 660 ± 15 nm or 640 ± 30 nm. Internal dye concentration was estimated at between 0.5 and 1.0 mM for most experiments. At this concentration, the buffering effect of the dye did not change the natural oscillatory behavior of the neuron as recorded intracellularly or the motor pattern as monitored by extracellular nerve recordings. However, experience with other systems (18) suggests that the recovery time-course of calcium transients should be slowed by this amount of dye.

Absorbance changes were recorded simultaneously at 96 positions by using a 10×10 photodiode array. This detection system was used essentially as described (17, 18). Briefly, the preparation was mounted on the stage of a Zeiss Universal microscope. Using a $\times 25$ water-immersion lens (Leitz 519706) the ganglion was imaged onto the photodiode array; each element detected the absorbance change in a $60 \times 60 \mu m$ pixel. The absorbance changes were recorded by using a low-pass filter with a time constant of 10 msec. The DC component of each record was subtracted by using a sample and hold circuit, and the resulting signal was amplified, multiplexed, and digitized with 12-bit accuracy. Sample points were taken every 8–30 msec. Typically 25–50 trials were averaged to give the signals we measured. After taking the arsenazo III data, the cell was filled with the dye Lucifer yellow and was photographed *in situ*. Cell shape was reconstructed superimposed on the map of the photodiode signals to correlate cell geometry with sites of absorbance change.

RESULTS

Fig. 1 shows a typical result when a neuron was depolarized with injected current sufficient to elicit a burst of action potentials. Absorbance increases at 640 nm were recorded from most parts of the neuron: soma, major process, and fine neuropil processes. The absorbance changes begin close to the start of the current pulse and continue to increase, reaching a peak 50–60 msec after the end of the pulse. About 20 msec of this delay is due to the time constant of the electronics and another 10 msec may be due to the sampling interval we used. In agreement with previous workers (17, 25–27), we interpret the arsenazo III absorbance changes as reflecting a stimulus-induced increase in internal free calcium concentration and the subsequent return to equilibrium as a consequence of internal buffering, sequestration, and active extrusion of calcium. Because the effective diffusion constant of calcium is slow ($D = 3 \times 10^{-7}$ cm^2/sec; ref. 28) and because the changes are recorded almost simultaneously over all parts of the cell, there is not enough time for calcium to spread throughout the cell by diffusion from one or a few restricted sites. Thus, the absorbance changes must reflect calcium entering the arsenazo-filled intracellular space through a voltage-dependent mechanism (such as calcium channels in the plasma membrane) located in all parts of the neuron. Since the absorbance change is linear with a change in internal free calcium (25, 29) and the stimulus length is short compared to the recovery time, the amplitude of the signal at each position is proportional to the amount of calcium that entered the cell through the surface under each pixel element. If we assume that the calcium entered from the extracellular space through voltage-gated calcium channels, then the amplitude of the absorbance changes can be interpreted as proportional to the number of calcium channels in the membrane, if we assume that the potential change during the burst was the same all over the cell. This assumption is
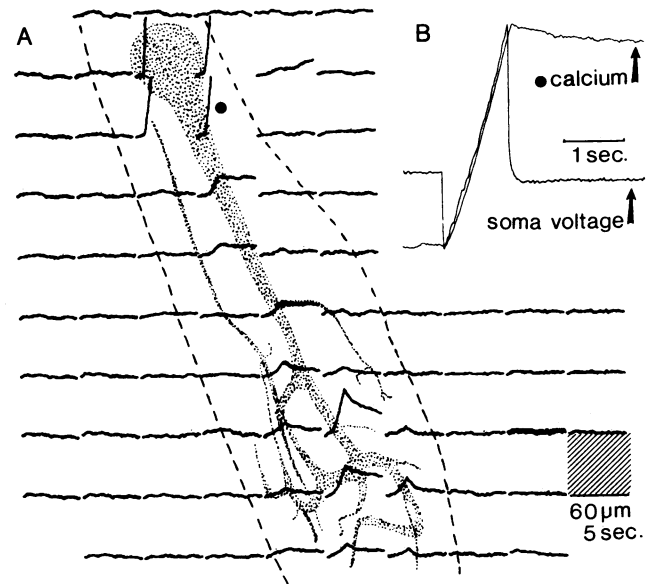

FIG. 1. Simultaneously recorded changes in absorbance shown for most of the 96 positions over the stomatogastric ganglion (dashed lines outline the ganglion) while a gastric neuron soma was depolarized by +10 nA for 1 sec, causing its axon to fire a burst of action potentials. Each short trace records the absorbance change during a 5-sec period for a single pixel element ($60 \times 60 \mu m$) as indicated by the cross-hatched square over the calibration bar. Vertical scale is arbitrary but is the same for all elements. Absorbance increases are found at all positions over the neuron except the axon (as determined by the post-experiment Lucifer yellow fill of the cell) and not over other positions in the ganglion. The absorbance increases during the burst and recovers to baseline with a time constant of several seconds. Several traces over the soma are off scale and only the rising phases are shown. The small oscillations in some of the elements not over the cell are probably due to movement artifacts at the edge of the ganglion or to debris in the recording chamber; 50 sweeps were averaged with an interval of 15 sec between sweeps. (*Inset*) Simultaneous electrical recordings from the soma with an unbalanced bridge electrode and the absorbance signal recorded from the element near the soma is indicated by a dot. Vertical scale is normalized so both signals show the same peak-to-peak excursion. Signal averaging process has smoothed over the action potentials on the electrode recording.

likely to be true in neurons when the action potential actively invades the soma (18), but it is unlikely to be valid for the neurons of the stomatogastric ganglion that do not have overshooting spikes in the neuropil or soma (7, 8). Therefore, the large signals recorded near the region of major process branching of this cell may reflect a larger local depolarization and/or a greater channel density in this region. Little absorbance change was detected over the axon, probably because of a low density of calcium channels there, but possibly because of poor diffusion of arsenazo III into the axon.

Fig. 2 shows a similar montage from a ventricular dilator (VD) neuron. In this preparation, the pyloric cycle frequency was 2 Hz. During each cycle, the VD went through an oscillation in membrane potential of from 5 to 16 mV. Small spikes were recorded in the soma during the peak of each oscillation. The montage shows that absorbance changes, reflecting voltage-dependent calcium entry, are recorded all over the cell (except for the axon) when stimulated intrasomatically. However, when the cell was driven by its natural oscillation and not by the electrode in the soma, absorbance changes were detected above noise level in only those locations indicated by a dot. These locations include much of the neuropil, but not the soma. The lack of signal in the soma was not due to the dilution of entering calcium by the large volume of the soma compared to the small volume of the
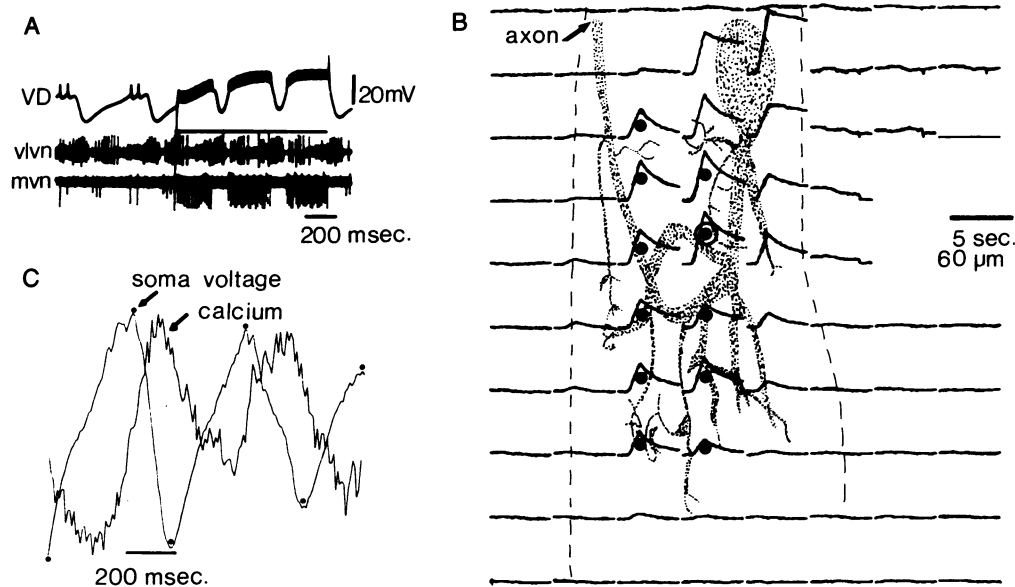
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FIG. 2. Calcium signals in neuropil of a pyloric VD neuron. (*A*) Simultaneously recorded intracellular and extracellular voltages. Note that the (retouched) extracellular spikes seen on the median ventricular nerve recording (mvn), correspond 1:1 with the action potentials at the peak of the oscillations. During the second half of the trace, indicated by the line, the VD soma was depolarized by the injection of +3.5 nA of current through the unbalanced bridge circuit. (*B*) Simultaneously recorded absorbance changes at many positions when the VD neuron was stimulated as shown in *A* to fire intense bursts of action potentials. Similarly to the cell in Fig. 1, absorbance changes are found in all locations over the neuron except the axon; 50 sweeps were averaged with an interval of 10 sec between sweeps. Dots indicate those locations where absorbance oscillations were detected above noise level when the cell spontaneously oscillated. (*C*) Simultaneously recorded electrical and absorbance oscillations. Electrical recording was made from the soma; absorbance change was recorded from the element marked with a circle; 200 sweeps were averaged. During each 2-sec sweep, measurements were made every 8 msec of the soma voltage and of the output of each element of the photodiode array. The sweeps were initiated at the trough of the VD soma voltage oscillation, causing the first burst in each sweep to occur with approximately constant latency. The latencies to the second and third bursts in a sweep were more variable because of spontaneous fluctuations in the burst period. Thus, the averaged data from later bursts during a sweep were always smaller and broader because of asynchrony in the data. Vertical scales are normalized and arbitrary as in Fig. 1 (*Inset*).

neuropil processes, because the signal size is expected to be proportional to the magnitude of calcium entry and not to the average value of the concentration change. In fact, we might expect that for a uniform channel distribution and membrane depolarization the oscillation would be more easily detected in the soma because the soma completely covers a pixel.

The absorbance increase reached a peak 100–150 msec after the peak of the potential change (Fig. 2*C*), resulting in a phase shift of >80°. This phase shift was the same at all locations where the absorbance oscillations were detected.

Fig. 3 shows similar results from another cell. The electrical recordings that identify this neuron as a lateral pyloric (LP) neuron are shown in Fig. 3*A*. In this cell, the amplitude of the electrical oscillation was greater, having a flat top with a strong burst of action potentials at the peak. Fig. 3*C* shows the simultaneously recorded electrical oscillation and the absorbance signal at a position in the neuropil. This cell was particularly interesting because the calcium oscillations were detected in the neuropil without averaging (Fig. 3*B*), indicating that the amplitude of the oscillation must be substantial. Yet, in the soma, an average of 25 sweeps revealed little oscillation above noise level (Fig. 3*B*).

Comparing the electrical and calcium signals in Fig. 3*C*, we see that the rising phase of the calcium signal begins during the rising phase of the electrical oscillation. The signal continues to increase during the burst but with a decreasing rate of change, reaching a peak ≈100 msec after the end of the burst.

DISCUSSION

Many of the neurons we examined showed transient calcium concentration increases in all regions of the cell (except the axon) when stimulated intrasomatically. In some experi-

ments, particularly those during the earlier phase of our work, we found cells that had signals confined to the soma, or with no signals. In these cases, it is likely that the dye did not diffuse throughout the cell or that the cell was damaged in some way. While we have yet to complete an exhaustive inventory of all the cells in the ganglion, we can conclude that calcium channels are distributed over all nonaxonal regions of some neurons. The density of channels in different regions could not be further analyzed with this method because electrical potentials do not spread with uniform amplitude over stomatogastric neurons (7, 8). In the barnacle (18), where this assumption can be justified in some cells, the calcium channel density was found to be higher in processes and presynaptic terminals than in axons.

The wide distribution of calcium channels in neuropil regions of stomatogastric neurons is consistent with anatomical measurements (5, 6), which suggest that synaptic contacts are widely distributed in the neuropil. However, the finding of significant channel density on the soma and major processes suggests that not all calcium channels are associated with release sites.

Of particular interest is the finding that calcium oscillations can be found over much of the neuropil and that for a given neuron the period and phasing of the oscillations are constant throughout the neuropil but are phase shifted with respect to the membrane potential oscillation. Thomas and Gorman (19), using a single detector, found oscillations in the soma of R15 in the abdominal ganglion of *Aplysia californica*, but they made no observations on the neuropil. The most likely explanation for their detection of somatic calcium oscillations and their absence in stomatogastric neurons is that somatic action potentials are of large amplitude, overshooting 0 mV in R15. In contrast, the action potentials in stomatogastric cells are only a few millivolts high and, even riding on top of
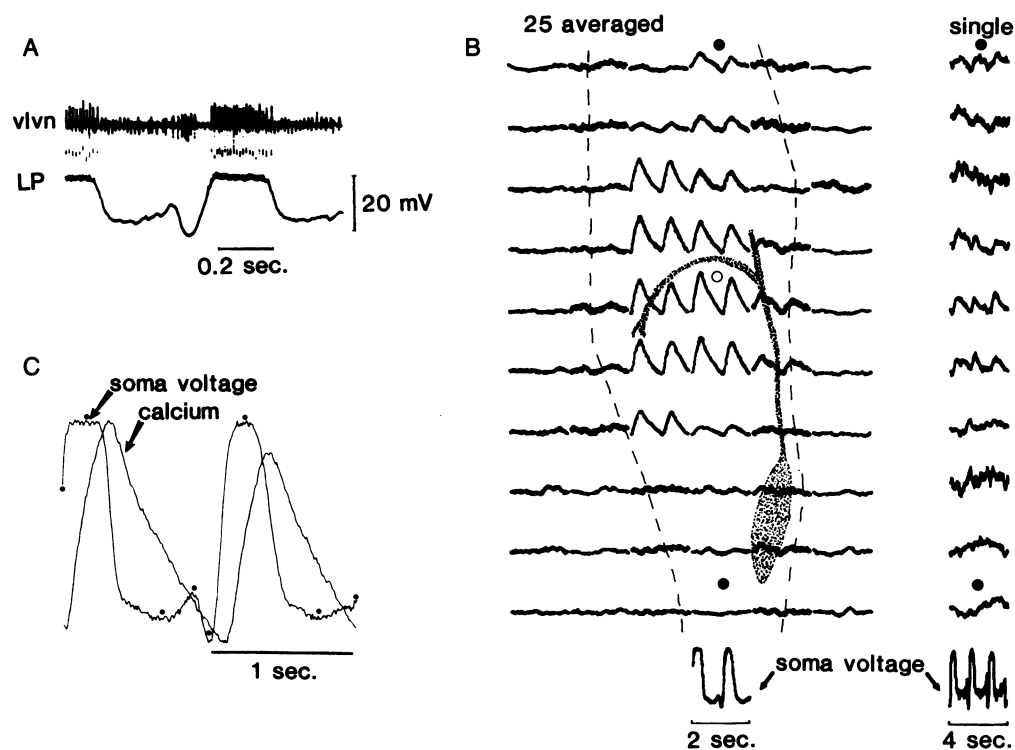
FIG. 3. Natural calcium oscillations in the neuropil of a pyloric LP neuron. (A) Intracellular and extracellular recording from the ventral lateral ventricular nerve (vlvn). Note the large amplitude of the slow electrical oscillation and the small amplitude of the somatic spikes. (B) Absorbance oscillations recorded at many positions superimposed on a drawing of the stomatogastric ganglion (dashed outline) and of the LP neuron. In this cell, only the soma and major process are shown because the Lucifer dye did not fully penetrate the cell. Large oscillations are seen over the region of the neuropil where the LP fine processes are probably located; 25 sweeps were averaged for those records. Column on right shows data taken from a single 4-sec sample showing that, for this cell, the oscillations could be seen without averaging. Dots are markers for the same grid elements in each map. Each grid element is 60×60 μm. (C) Superimposed soma voltage and neuropil calcium oscillations from the element indicated by an open circle in B. Vertical scales are normalized as in Figs. 1 (*Inset*) and 2C.

the slow oscillation, reach a level considerably negative to 0 mV. In the cell bodies of gastropod molluscs, significant optical signals are usually only detected when the membrane is depolarized to -20 mV (25, 26). If the same threshold applies to our cells, the peak somatic depolarization would not be large enough to cause measurable changes in the internal calcium concentration. No studies of calcium currents in isolated cell bodies of stomatogastric neurons have yet been made. However, in crustacean cardiac ganglion neurons, the soma is actively involved in the generation of driver oscillations and has a calcium current with a voltage threshold of about -50 mV (30).

On the other hand, the rising phase of the calcium oscillation in the LP neuron appears to begin before the burst of action potentials in that cell, and the calcium signal continues to increase after the end of the burst. These data indicate that substantial calcium entry occurs during the slow wave and that it does not require the larger amplitude of the action potentials to open the calcium channels. This result is consistent with the observation of graded synaptic transmission without action potentials in this system (31, 32). In *Panulirus interruptus*, LP neurons have a threshold for detectable transmitter release at -60 mV (4). A threshold for voltage-dependent calcium influx of about -50 to -60 mV would be consistent with our data from *Cancer* neuropil. However, it is difficult to reconcile with the soma data which resembles that of mollusc. One possible explanation of these results is that *Cancer* is not like *Panulirus*. If *Cancer* neuropil processes were more depolarized than the soma or if they had a larger depolarizing component to their oscillation, then both soma and neuropil could have mollusc-type calcium channels. However, this explanation may require more voltage attenuation between soma and neuropil than is realistic (9,

10). A second possible explanation is that the properties of the calcium channels in the soma are different from those in the neuropil, with the latter having a lower threshold for opening. Co-occurrence of low-threshold (inactivating) and high-threshold (noninactivating) calcium channels has been reported in several types of vertebrate neuron (33–37). There also can be more than one type of calcium channel in invertebrate neurons (refs. 38 and 39; but see also ref. 37).

The origin of the phase shift between the oscillating membrane potential and the absorbance change may lie in the voltage dependence of the calcium channels. Fig. 3C shows that the absorbance signal begins to increase during the depolarizing phase of the soma membrane potential. It continues to increase through the peak of the electrical oscillation and during the first part of its falling phase. If the threshold for calcium entry in the neuropil is at a level about two-thirds the amplitude of the voltage oscillations, and the calcium channels do not inactivate over several hundred milliseconds, then calcium would enter during the entire time that the membrane potential was above the threshold level, and absorbance oscillations of the kind we observed would be expected. Since the internal calcium concentration is determined by both entry and removal of calcium from the arsenazo III-intracellular space, any change in the removal rate will also modify the signal. If the removal sites are located nonuniformly within the cytoplasm or are dependent on past history or are saturable, then the removal during continuing oscillations in internal calcium may differ from that in response to a pulse of calcium. A change in removal could then affect the shape and phase shifts of the absorbance signal. The cable properties of the cell are not relevant in this situation because the phase shift is constant throughout the neuropil and is in the wrong direction for a cable effect.

Thomas and Gorman (19) did not describe a phase shift, but their apparatus had a 1 Hz filter, which could have obscured a delay of the magnitude observed in our cells.

It is interesting to try to estimate the magnitude of the calcium concentration changes occurring in the neuropil during these natural oscillations. In the LP neuron, the change in absorbance during one cycle for one pixel element over the neuropil was $\Delta A = 1/6500$. Dye concentration was estimated at ≈ 1.0 mM as judged by the color of the soma. We will assume that the dye is distributed uniformly. The change in absorbance as a function of free calcium change has been calibrated by Gorman and Thomas (20). They also point out that this calibration is sensitive to dye concentration. The largest unknowns in the calculation are how much area the neuropilar processes occupy in an individual pixel and what are the typical thicknesses of these processes. Unfortunately, we did not have a good Lucifer yellow fill of the cell with the large oscillations (Fig. 3). If we use typical values from other fills, then we would estimate that the processes occupy at most 25% of the pixel area with a typical thickness of 3 μm. Using these values, we calculate that the peak change in free calcium is at least 0.35 μM. This is to be compared with the estimated value of 0.1 μM for the resting calcium level in most neurons (40).

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