Highly Localized Ca^{2+} Accumulation Revealed by Multiphoton Microscopy in an Identified Motoneuron and Its Modulation by Dopamine

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Calcium is essential for synaptic transmission and the control of the intrinsic firing properties of neurons; this makes Ca^{2+} channels a prime target for neuromodulators. A combination of multiphoton microscopy and voltage-clamp recording was used to determine the localization of voltage-dependent Ca^{2+} accumulation in the two pyloric dilator (PD) neurons of the pyloric network in the spiny lobster, *Panulirus interruptus*, and its modulation by dopamine. We monitored $[\text{Ca}^{2+}]_i$ in fine distal branches in the neuropil $>350\ \mu\text{m}$ below the surface of the ganglion during controlled voltage steps in voltage clamp. Ca^{2+} accumulation originated mostly from small, fairly rare, spatially restricted varicosities on distal neuritic arborizations. Ca^{2+} diffused from these point sources into adjacent regions. Varicosities with similar morphology in the PD neuron have

been shown previously to be sites of synaptic contacts. We have demonstrated in earlier studies that dopamine inhibits activity and greatly reduces synaptic transmission from the PD neuron. In $\sim 60\%$ of the varicosities, the voltage-activated Ca^{2+} accumulation was reduced by exogenous dopamine (DA) ($10^{-4}\ \text{M}$). DA decreased the peak amplitude of Ca^{2+} accumulation but had no effect on the rise and decay time. We conclude that DA reduces chemical synaptic transmission from the PD neurons at least in part by decreasing Ca^{2+} entry at neurotransmitter release sites.

Key words: calcium; crustacean; central pattern generator; dopamine; motoneuron; multiphoton microscopy; neuromodulation; stomatogastric ganglion; two-photon microscopy

Calcium plays a critical role in both synaptic transmission and the control of intrinsic firing properties of neurons, such as rhythmic bursting and bistability (Augustine et al., 1987; Hille, 1992; McCleskey, 1994; Zhang and Harris-Warrick, 1995; Fisher and Bourque, 1996; Zucker, 1996). As a consequence, intracellular Ca^{2+} levels are carefully controlled in neurons and are a major target of neuromodulators, which act to change the firing properties of neurons and their synaptic interactions. This control is usually aimed at modulation of voltage-dependent Ca^{2+} currents (Berger and Takahashi, 1990; Zhang and Harris-Warrick, 1995; Karunanithi et al., 1997).

We have used a combination of voltage clamp and multiphoton microscopy (MPM) (Denk et al., 1990; Xu et al., 1996; Denk and Svoboda, 1997) to analyze changes in voltage-evoked Ca^{2+} accumulation in fine neurites of the pyloric dilator (PD) neurons in the pyloric network of the stomatogastric ganglion of the spiny lobster (*Panulirus interruptus*). The pyloric network is a small central pattern generator network that has served as a model for neuromodulation of neural networks at the cellular and synaptic level of analysis (Harris-Warrick et al., 1992). The two PD neurons, along with the anterior burster neuron, form the pace-maker group that sets the cycle frequency (Johnson and Hooper, 1992; Ayali and Harris-Warrick, 1999). In earlier studies, rhythmic

oscillations of calcium were seen in the neuropil but not in the somata or axons of these unipolar neurons (Graubard and Ross, 1985; Ross and Graubard, 1989). These recordings were made with a photodiode array using Arsenazo III and required signal averaging to detect the small Ca^{2+} signals. However, they showed that there are regional differences in the intracellular accumulation of Ca^{2+} in these neurons.

Bath application of the neuromodulator dopamine (DA) reconfigures the pyloric central pattern generator by enhancing activity in some neurons and reducing activity in others (Anderson and Barker, 1981; Eisen and Marder, 1984; Flamm and Harris-Warrick, 1986). In the PD neurons, dopamine causes a hyperpolarization and reduction of the number of action potentials per cycle, leading to a reduction in the overall pyloric cycle frequency (Flamm and Harris-Warrick, 1986). In addition, DA reduces and often abolishes synaptic transmission from PD synapses (Johnson and Harris-Warrick, 1990). The cellular mechanisms underlying the inhibition of the PD neurons by DA are only partially understood. The hyperpolarization and reduction in firing frequency appear to result from dopamine enhancement of two voltage-dependent K^+ currents, the transient K^+ current (I_A), and the calcium-activated K^+ current ($I_{\text{O}(\text{Ca})}$) (Kloppenburg et al., 1999). Whereas modulation of these K^+ currents may also contribute to reducing release from PD nerve terminals, other ionic currents, including Ca^{2+} currents, could be selectively modulated at nerve terminals in a way not easily detectable by voltage clamp from the soma (Johnson et al., 1999).

Using MPM Ca^{2+} imaging combined with voltage clamp, we have performed experiments to determine (1) where in the PD neuron voltage-induced Ca^{2+} accumulation occurs, and (2) whether Ca^{2+} accumulation at these sites is modulated by DA.

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Figure 1. Calcium Green-1-loaded PD neuron. *A*, The image is a projection of 200 sections taken with a 20×0.5 NA objective and a $2\ \mu\text{m}$ axial step size. To ensure high contrast, the laser intensity was adjusted as needed during the acquisition of the z-series using the Pockels Cell. Excitation was at 800 nm, and the non-descanned emission was collected through a 575DF150 bandpass filter. Punctate fluorescence spots are autofluorescence from unknown objects in the neuropil. *B*, Projected side view of the same data set as in *A*, demonstrating the depth of recording capability.

MATERIALS AND METHODS

California spiny lobsters, *Panulirus interruptus*, were obtained from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained up to 4 weeks in artificial seawater at 16°C until use. Calcium Green-1, Indo-1, Fluo-3, and fura-2 were obtained from Molecular Probes (Eugene, OR). Other chemicals were obtained from Sigma (St. Louis, MO).

Preparation. Animals were anesthetized by cooling in ice for at least 30 min before dissection. The stomatogastric ganglion (STG), along with its motor nerves and the associated commissural and esophageal ganglia, was dissected from the animal (Selverston et al., 1976) and pinned in a Sylgard-coated dish. The preparation was superfused continuously (3 ml/min) with saline (16°C) of the following composition (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl_2 , 3.9 Na_2SO_4 , 10.0 MgSO_4 , 2 glucose, and 11.1 Tris base, pH 7.35 (Mullony and Selverston, 1974). Extracellular recordings were made from identified motor nerves using bipolar pin electrodes insulated by Vaseline. After desheathing the STG, individual somata were impaled with glass microelectrodes ($10\text{--}25\ \text{M}\Omega$; 2.5 M KCl) and identified using three criteria: (1) a 1:1 correspondence of action potentials recorded intracellularly in the soma and extracellularly from an identified motor nerve; (2) characteristic phasing and synaptic input during the pyloric motor pattern; and (3) characteristic shape of the membrane potential oscillations and action potentials in the pyloric rhythm.

After electrophysiological cell identification, the recording chamber was transferred from the identification rig to the imaging set up. The recording chamber was mounted on the modified temperature-controlled stage of a modified Olympus AX-70 upright microscope (Olympus Opticals, Melville, NY). The preparation was constantly superfused with saline (3 ml/min) at 16°C .

The PD neurons were iontophoretically loaded with Calcium Green-1 for most experiments or Indo-1 to determine the absolute resting Ca^{2+} concentration. Both dyes (2 mM in H_2O) were injected with hyperpolarizing current until fine neuritic arborizations were visible (Fig. 1). For

Calcium Green-1, the injection was standardized to $-10\ \text{nA}$ for 20 min to minimize dye concentration differences between experiments. Using these parameters, the injected dye had no immediate effect on the firing properties of the neuron, which were monitored in a rhythmically active preparation during brief interruptions of the current injection.

To remove chemical synaptic input from the dye-loaded PD, 5×10^{-6} M picrotoxin (PTX) (Bidaut, 1980) was added to the bathing solution. Modulatory inputs from other ganglia were eliminated with a 10^{-4} M tetrodotoxin (TTX) block of the stomatogastric nerve, the sole input nerve to the STG. To improve voltage control of distal neurites, we blocked other conductances than Ca^{2+} with the following compounds. Sodium currents (I_{Na}) were blocked by TTX (10^{-7} M). A hyperpolarization-activated inward current (I_h) was blocked by CsCl (5×10^{-3} M). TEA (2×10^{-2} M) was used to block $I_{\text{K(V)}}$ and $I_{\text{O(Ca)}}$. To compensate for changes in osmolarity, the NaCl concentration was reduced. Although 4-aminopyridine (4-AP) (4×10^{-3} M) has been shown to be a selective blocker of I_A in other STG neurons (Graubard and Hartline, 1991; Tierney and Harris-Warrick, 1992), it induced a large and reversible leak current in PD (Kloppenburg et al., 1999) and thus was not used routinely. I_A was instead eliminated by holding the PD neuron at $-45\ \text{mV}$ at which I_A is almost completely inactivated (Baro et al., 1997; Kloppenburg et al., 1999). Calcium currents (I_{Ca}) were blocked by CdCl_2 ($2\text{--}6 \times 10^{-4}$ M) or 0 Ca^{2+} (replaced by Mg^{2+}) saline.

Voltage clamp of synaptically isolated PD neurons. Synaptically isolated PD neurons were impaled with two electrodes for voltage recording and current injection (10 $\text{M}\Omega$; 2.5 M KCl or 2.5 M K-acetate with 2×10^{-2} M KCl). The cell was voltage clamped using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). Voltage protocols were generated with the aid of pCLAMP6 and a Digidata 1200A interface (Axon Instruments) running on a personal computer.

Dopamine application. Dopamine (10^{-4} M) was bath-applied at 3 ml/min into a bath volume of 3 ml. The threshold for detectable inhibition of the PD neuron by DA is 10^{-5} M, and a maximal effect is observed at 10^{-4} M (Flamm and Harris-Warrick, 1986).

Imaging. The combined multiphoton microscope–electrophysiology setup consisted of a Spectra Physics Tsunami Ti:S laser with a 20 W argon pump (Spectra Physics, Mountain View, CA), a retro-fitted Bio-Rad (Hercules, CA) MRC-600 scanbox, and a custom built, fixed stage Olympus AX-70 upright microscope. A Hamamatsu (Bridgewater, NJ) HC125-02 photomultiplier tube placed directly above the objective lens was used to collect the non-descanned emission (500–600 nm). The beam intensity was controlled using a ConOptics (Danbury, CT) model 350–50 Pockels Cell, which also blanked the laser during fly-back (in between scan lines), eliminating unnecessary excitation of the preparation.

PD neurons loaded with Calcium Green-1 were imaged with 800 nm excitation (~ 100 fsec before the objective lens) through a 20×0.5 NA or 40×0.8 NA water immersion objective lens. Indo-1-loaded cells were imaged at 720 nm, and emission was collected at 390 nm (390/65 band-pass filter) and 495 nm (495/20; Chroma Technology Corp., Brattleboro, VT). *In vivo* calibrations were performed for the Indo-1 measurements and to determine the maximal possible F/F_0 with Calcium Green-1. Saturating and 0 Ca^{2+} levels were obtained by bathing loaded cells with the ionophore 4Br-23187 (~ 10 mM), followed by perfusion with normal saline and then 0 Ca^{2+} buffer and injection with EGTA. Calcium transients were acquired using line scans at a rate of 2 or 4 msec per line. Voltage-clamp data were simultaneously recorded on the second channel of the Bio-Rad scanner during the line scans to synchronize the start of the voltage pulse with the Ca^{2+} signal.

Data analysis. Data extraction and fitting were performed by laboratory written software. Pixel values were extracted from the line scan images (Fig. 2*D,E*) along the time axis in the area of interest (averaged across the spatial axis). The simultaneously acquired membrane potential was analyzed by the software (plot superimposed on Fig. 2*C*) to determine the starting point of the voltage pulse, ensuring synchronization between the Ca^{2+} data and the start of the voltage pulse. Data points corresponding to the Calcium Green-1 signal during the voltage pulse were fit to a modified Chapman function:

$$(1) \quad \frac{F}{F_0} = A(1 - e^{-t/\tau})^{B+1}$$

where the amplitude A is equal to $(F_{\text{max}} - 1)$, τ is rise time, and B is a unitless parameter that is 0 for the case of line scan data taken at a point of Ca^{2+} influx at the membrane and gets larger as the measurement

Figure 2. Data acquisition and analysis of voltage-induced calcium transients. *A*, A projected image of 30 optical sections from a part of a Calcium Green-1-loaded PD neuron with a region containing several active varicosities outlined by the yellow box (20×0.5 NA objective). *B*, A single optical section of the boxed region in *A* taken at higher magnification (40×0.8 NA) with the line scan position marked with a yellow line. Branches that are only partly or not in the optical section are accordingly dimmer or not visible at all. *C*, The “image” of the soma voltage collected simultaneously with the Ca^{2+} indicator signal to ensure accurate synchronization of the start of the voltage pulse and fluorescence data analysis. Plot shows the soma voltage trace taken from the pixel values in *C*. *D*, Line scan image taken at the points indicated in *B*. The temporal resolution was 4 msec per line. The time scale is the same as in *C*. *E*, Data extracted from the image in *D*, along the time axis. Each point in time is the average of five pixel values across the spatial axis from the region marked by arrows in *B/D*. The dashed lines show the voltage pulse duration (-45 to 0 mV for 200 msec). The data during the voltage pulse was fit to Equation 1 (see Materials and Methods); the decay of fluorescence was fit to a single exponential decay model [$F(t) = F_{\max} \exp(-t/\tau_{\text{decay}})$].

location is moved further away. Equation 1 has the property that it tends to a simple exponential rise as the delay parameter B , and, therefore the delay time, approaches 0. Traces were first normalized so that $F_0 = 1$, hence the offset of 1 in the above equation. This equation was applied to determine a phenomenological rise time and delay parameter in a consistent manner that could be easily automated in software using the Levenberg–Marquardt algorithm. Based on the inflection point of Equation 1, a delay time can be calculated as $\tau \ln(B + 1)$, which is the time at which a function of the form $1 - \exp(-t/\tau)$ would intercept the time axis if it were “moved over” so that at long times it exactly coincided with Equation 1. This is shown graphically in Figure 3 in which Equation 1 is plotted with $\tau = 30$ msec and $B = 15$. The delay time would be 83 msec and corresponds to the time at which a simple $1 - \exp(-t/30 \text{ msec})$ response would have to start to fit the data at long times as it tends to equilibrium. The fluorescence signal after the voltage pulse was fit to a single exponential decay model using the same analysis software.

Statistical analysis. Student’s t tests were used to assess the significance of differences between mean values of parameters measured under control conditions, during dopamine application, and after washing in dopamine-free saline. A Bonferroni correction was used to adjust for repeated t tests, and significance was accepted at $p = 0.025$. Throughout this paper, all calculated ranges are reported as the SD of the mean.

RESULTS

Measurement of voltage-induced Ca^{2+} influx in PD neurons

Of the Ca^{2+} indicators tested (Fluo-3, Indo-1, fura-2, and Calcium Green-1) we found Calcium Green-1 to have the most suitable properties for Ca^{2+} imaging in the STG, and we used it in all experiments in which voltage-dependent Ca^{2+} accumula-

Figure 3. Delay time definition. For a given value of the rise time τ , a delay time can be obtained from the delay parameter B in Equation 1 as $\tau \ln(B + 1)$ that is equal to the time at which a single exponential rise $[1 - \exp(-t/\tau)]$ would have to intercept the time axis (i.e., start) to converge to Equation 1 at long times. The solid line is Equation 1 (without the offset of 1) plotted with values of $\tau = 30$ msec and $B = 15$; the dotted line is the function $1 - \exp(-t/30 \text{ msec})$ plotted starting at the delay time of Equation 1 ($30 \ln(16) = 83$ msec).

tion was measured. It could be loaded reasonably rapidly into the large neurons of the STG, did not leak out over the course of the experiment, and had a sufficient fluorescence intensity change during a single voltage pulse to produce high signal-to-noise data. All dyes examined exhibited binding to internal proteins and membranes, reducing the maximum change in fluorescence possible with a given calcium influx. However, Calcium Green-1-loaded (and fura-2) cells appeared to have a lower fraction of bound, inactive dye. The parameters for dye injection were optimized empirically to provide good signals with minimal effect on the immediate physiological properties of the PD neurons, which were monitored during loading in a rhythmically active preparation. The Calcium Green-1-labeled PD neuron was isolated from chemical synaptic input by bath application of PTX (5×10^{-6} M). To improve the voltage clamp by reduction of electrotonic decay (Yuste et al., 1994), all identified conductances other than Ca^{2+} were blocked by bathing the preparation in TTX (10^{-7} M), CsCl (5×10^{-3} M), TEA ($2\text{--}10 \times 10^{-2}$ M), and in some recordings 4-AP (4×10^{-3} M). Imaging was not started until ~ 1 hr after loading to allow for a uniform distribution of dye within the neuron and time for the bath-applied inhibitors to take effect.

By spectrofluorometric measurement, we determined that ~ 120 fmol of Calcium Green-1 is iontophoresed out of the electrode during 20 min at -10 nA (our standard dye loading protocol). Although PD cell volumes vary from ganglion to ganglion, we estimated an average volume of $\sim 8 \times 10^5 \mu\text{m}^3$ (800 nl) based on volumetric analysis of several different image stacks of dye-filled PD neurons. From the amount of dye injected and the cell volume, we estimated that the total concentration of Calcium Green-1 was $\sim 150 \mu\text{M}$ in our experiments. Measurements of the fluorescence intensity of iontophoretically loaded PD neurons before and after the addition of a calcium ionophore showed that $\sim 70\%$ of the total dye concentration was available to dynamically bind Ca^{2+} (i.e., not bound to internal membranes or proteins), relative to the expected fluorescence change from resting level to saturation *in vitro*. From this measurement, we estimated the concentration of “active” Calcium Green-1 in the PD neurons to be $\sim 100 \mu\text{M}$. All preparations were loaded in the same manner, and we assumed that any measured differences in Ca^{2+} influx, kinetics, and accumulation between different experiments was not attributable to variations in indicator concentration.

The resting Ca^{2+} concentration of PD neurons was measured

by iontophoretic injection of the emission ratiometric calcium indicator Indo-1. Although internal binding of Indo-1 was severe, limiting the *in vivo* response of the indicator, we calculated a resting level of 97 nM at a holding potential of -45 mV, assuming a k_d of 250 nM (Grynkiewicz et al., 1985). The actual *in vivo* k_d is not known in this system and is a potential source of error in this measurement. Internal binding of the dye to cellular components reduced the measurable emission ratio change during the *in vivo* calibration procedure (0 Ca^{2+} to saturated dye) to $\sim 20\%$, which introduces further error from a reduced dynamic range. Using Calcium Green-1 fluorescence, only relative changes in $[\text{Ca}^{2+}]$ (usually F/F_0) are reported; however, the peak values of Ca^{2+} reached during the depolarizing voltage pulses can be estimated. Using a resting level of ~ 100 nM Ca^{2+} , the *in vivo* minimum (0 Ca^{2+}) and maximum (saturated dye) fluorescence intensities of a typical Calcium Green-1-loaded cell and a k_d of 190 nM, the peak $[\text{Ca}^{2+}]$ reached during a voltage pulse to 0 mV was ~ 400 nM. As with the Indo-1 measurements, the *in vivo* k_d of Calcium Green-1 in this system is not known and is a potential source of error in this estimate.

The three dimensional structure of the Calcium Green-1-loaded PD neuron was visualized with high spatial resolution to a depth of several hundred micrometers in the living ganglion. Sites of Ca^{2+} influx imaged in this study were usually between 150 and 250 μm below the surface of the preparation, with occasional regions of interest deeper than 350 μm (see Fig. 5A). To reconstruct the cell morphology, a full z-series of each investigated neuron was usually collected after the physiological measurements (Fig. 1). We could recognize all the typical morphological features of the PD neuron that were originally described by King (1976b). A single primary process leaves the soma and extends into the neuropil, where it eventually becomes the axon. Within the neuropil, a number of secondary processes branch from the primary process and then branch into higher order processes. Localized on these finer processes are irregularly shaped, enlarged varicosities, which have been demonstrated by electron microscopic studies to be either presynaptic or postsynaptic sites (King, 1976a; see Discussion).

To stimulate Ca^{2+} accumulation, the soma membrane potential was typically voltage clamped from a holding potential of -45 to 0 mV for 200 msec. The data acquisition and analysis is demonstrated in Figure 2. To maximize temporal resolution, we used a line scan mode in which a single line is scanned successively at 2 or 4 msec intervals. The line scans were displayed sequentially to produce a spatiotemporal image (Fig. 2D). The membrane potential was simultaneously recorded on the second channel of scanning microscope to provide an accurate temporal synchronization of the membrane potential (Fig. 2C) and voltage-induced calcium transients (Fig. 2D). Intensity change over time was extracted from regions of the line scan images and fit to Equation 1 given in Materials and Methods (Fig. 2E).

Voltage dependence of the induced Ca^{2+} influx

Considering its size and complex morphology, perfect voltage control of the entire PD neuron is not expected when voltage clamped from the soma. However, using a combination of ion channel blockers (Yuste et al., 1994), we were able to sufficiently reduce the electrotonic decay from the cell body to very distal arborizations so that we could demonstrate a clear, reproducible voltage dependence of the Ca^{2+} signal. This is shown in Figure 4 in which 200 msec voltage pulses of increasing amplitude were applied from a holding potential of -45 mV. Ca^{2+} accumulation

Figure 4. Voltage dependence of the induced Ca^{2+} influx in a PD neuron. *A*, The three traces show the kinetics of the influx and decay with three different voltage pulse amplitudes marked on the plots (dashed lines represent the voltage pulse). The holding potential is -45 mV. The top trace (no change in voltage) is also shown to demonstrate that photobleaching of indicator dye did not occur during line scan acquisition. *B*, The maximum Ca^{2+} signal as a function of the voltage pulse amplitude. Voltage pulses (200 msec) of varying amplitude were applied to the soma, and the Ca^{2+} influx was monitored in active regions in the neuropil. Data from four different PD neurons are shown (open and closed circles and triangles).

can be detected starting at voltages more positive than -40 mV, and the maximum is reached ~ 0 to $+10$ mV. More depolarized voltage steps often led to irreversible loss of signal and were not applied. However, the voltage dependence of the Ca^{2+} accumulation in Figure 4 is similar to the voltage dependence of Ca^{2+} currents measured from the PD soma (Johnson et al., 1999) and other stomatogastric neurons (Hurley and Graubard, 1998). Although the absolute voltage might differ somewhat from the measured voltage because of imperfect space clamp of the distal neuritic compartments, these results demonstrate that we had reasonable voltage control of distal regions of the neuron. Hyperpolarizing prepulses (up to 1 sec at -100 mV) did not increase the Ca^{2+} signal.

The voltage-evoked Ca^{2+} accumulation was completely dependent on the extracellular Ca^{2+} . Extracellular solutions with low $[\text{Ca}^{2+}]$ (but not <10 – 20%) reduced the voltage activated Ca^{2+} accumulation reversibly ($n = 3$). When Ca^{2+} -free solution was applied, the Ca^{2+} response was completely abolished. However, the response usually did not recover, even after prolonged (>1 hr) return to normal saline. Extracellular application of $\geq 200 \mu\text{M}$ CdCl_2 also blocked the Ca^{2+} signal.

Localization of points of Ca^{2+} influx

An initial series of experiments was undertaken to determine the spatial distribution of voltage-evoked Ca^{2+} accumulation. Line scans were used to measure voltage-induced Ca^{2+} accumulation at various regions of the neuron, including the cell body and primary, secondary, and terminal arborizations. The maximal amplitude and time course of the voltage-evoked Ca^{2+} signal was not homogenous within the neuron. In general, the amount of voltage-stimulated Ca^{2+} accumulation decreased from distal to proximal with only small and slow voltage-induced Ca^{2+} accumulation in the soma and major primary processes, unless arborizations were located near the soma as in the cell shown in Figure 5C. Analysis of the time course and amplitude at different locations in the PD neuron indicated that the Ca^{2+} signal originates mostly from small, spatially restricted sites on neuritic arborizations. Our data suggest that voltage-activated Ca^{2+} influx is restricted to morphologically distinct regions of the neurite, which typically have the shape of enlarged varicosities along or protruding from the neurite (Fig. 5A,C; see Fig. 7A,B) or smaller “bulbs” clustered in regions of small branches (Figs. 2B, 5B). Our results correlate well with earlier findings in which regional variations of $[\text{Ca}^{2+}]$ in rhythmically active stomatogastric neurons were found (Graubard and Ross, 1985; Ross and Graubard, 1989). Using electron microscopy, King (1976a) showed that similar varicosities are specialized regions of chemical presynaptic or postsynaptic contacts usually containing more than one synapse. He did not detect presynaptic and postsynaptic sites in the same varicosity. The varicosities had diameters of up to $10 \mu\text{m}$ on neuritic processes otherwise only 1 – $3 \mu\text{m}$ in diameter. Our hypothesis that the presence of voltage-gated Ca^{2+} channels is primarily restricted to these regions is demonstrated in Figures 2 and 5. In both figures, localization is evidenced by differences in the delay in the onset of Ca^{2+} accumulation on neuritic arborizations measured by separate line scans taken a few micrometers apart along the neurite (Fig. 5B) or from the same line scan taken along a process (Figs. 2B–E, 5A). In Figure 2E, the rise time gets slower and the maximal Ca^{2+} level decreases with increasing distance from the region in which Ca^{2+} influx is assumed (Fig. 2E, compare 1, 2), indicating localized Ca^{2+} influx at site 2 (Fig. 2B,D). This is demonstrated again in Figure 5, A and B, in which only the rising phase of the Ca^{2+} signal is plotted. At regions along the neurite, but away from varicosities, a delay in the Ca^{2+} rise is clearly seen relative to the start of the voltage pulse (Fig. 5A,B). The delay in the rise was phenomenologically quantified using Equation 1 (see Materials and Methods), and the delay time increases with the distance of the line scan from the region of influx. Overall, diffusion *in vivo* is anomalous (Brown et al., 1999). Factors such as morphology and differences in intrinsic Ca^{2+} buffer concentrations will affect the observed kinetics. At short distances ($<2 \mu\text{m}$) from the influx point, diffusion appears very slow, perhaps because of localized buffering and spatial restriction (Svoboda et al., 1997) (see also Fig. 5). However, over distances more than a few micrometers from the assumed influx point, the delay time becomes a coarse, but useful, indicator of the distance from the actual Ca^{2+} source (Fig. 6A).

Rise-time time constants obtained by fitting the Ca^{2+} accumulation during the voltage pulse to Equation 1 ranged between 10 and 20 msec at points of presumed influx (defined by a delay parameter of <20 msec) (Fig. 6B). The rise times become slower as the distance from the point of presumed Ca^{2+} influx increases (Fig. 6B). Only data taken from line scans at the actual region of

Figure 5. Voltage-induced Ca^{2+} accumulation is highly localized in PD neurites. *A*, Image of a neuritic varicosity similar to those described by King (1976a) as a swelling on a process. The image in *A* is a single optical section of the region under investigation. A line scan image from the region is shown below the *x-y* image. The bars mark the corresponding regions. The extracted data from these regions during the voltage pulse are shown in *A1* and *A2* illustrating the delay that is seen at regions away (*A2*) from the site at which Ca^{2+} influx seems to occur (*A1*). The voltage pulse (0 mV; 200 msec; holding potential of -45 mV) starts at 0 msec. *B*, Process from a different PD neuron with varicosities branching off the neurite (similar to those shown in Fig. 2). The Ca^{2+} rise during the voltage pulse is shown from three different positions on the neurite and its attached synaptic structures (marked by the color-coded bars 1, 2, and 3). The Ca^{2+} accumulation during a 200 msec voltage pulse is plotted in *B1–B3*. The pulse starts at 0 msec. In *B1*, the plotted data are the average from the two regions indicated by the black bars at position 1 in *B*; both areas had similar rise kinetics and were averaged to reduce the noise. Voltage-gated Ca^{2+} channels seem to be located on the two bulbous varicosities in *B* (region 1), as evidenced by the fast rise without delay. Further away along the dendrite (sites *B2* and *B3*), the delay-to-rise markedly increased. *C*, A large varicosity near the soma of a PD neuron. The low-magnification (20×0.5 NA) image is a projection of 100 sections. Pixel values of the cell body were processed off-line (scaled to low values) to show the connection to the primary neurite. The inserted image is a high-magnification (40×0.8 NA) view of the varicosity. The plot below the images show the maximal value of F/F_0 attained during the voltage pulse as a function of distance (color-coded 1.8 micrometer steps) from the tip of the structure.

the Ca^{2+} source could be fit as a simple exponential rise [$1 - \exp(-t/\tau)$]; most line scans required a sigmoidal function with a delay to onset for an adequate description (Fig. 5*A,B*).

The apparent rate at which the Ca^{2+} signal dissipates after a voltage pulse in the STG can depend on several factors, including the rate of extrusion out of the neurite, uptake into internal stores, the internal buffering capacity, the relative fractions of mobile and immobile buffers, and the distance from the region of influx (Sala and Hernandez-Cruz, 1990; Neher and Augustine, 1992; Nowycky-Martha and Pinter, 1993). With discrete regions of influx separated by tens of micrometers, such as found in the STG neuropil, the “blurring” of the induced Ca^{2+} pulse caused

by diffusion is a primary factor in the slowing of the decay of the Ca^{2+} signal at points further away from the point of influx. This produces an increase in the decay time of the Ca^{2+} signal, the extent of which is further affected by the *in vivo* buffering capacity of the neurite. In the STG neuropil, the measured decay constants correlate with the distance of the measurement site from influx point. Figure 6*C* shows a plot of the decay constant against the delay time from 438 line scans performed in 11 different PD neurons. Although the linear correlation between these two parameters is weak, it is clear that, near the varicosity (at which the delay time is minimal), the decay rate of Ca^{2+} is fastest. The decay time at a distance from the assumed Ca^{2+} influx point

Figure 6. Correlation between the delay time and other experimental parameters. *A*, Delay time as a function of the distance from the presumed point of Ca^{2+} influx (defined by ~ 0 delay time) from four PD neurons that had regions of Ca^{2+} accumulation that were demonstrably isolated from other active varicosities along the same neurite. *B*, Correlation of the rise time with the delay time for 438 measurements taken from 11 different PD neurons. The rise time increases with the distance from the region in which Ca^{2+} influx is assumed as measured by the increased delay time (as expected from diffusion of Ca^{2+}). *C*, Correlation of the decay time with the delay time from 438 measurements taken from 11 PD neurons. Points near the region of fastest Ca^{2+} accumulation consistently exhibit the fastest apparent decay time. Decay rates are highly variable at longer distances from the presumed influx point.

varied greatly, depending on the particular morphology. Ca^{2+} influx from multiple distant varicosities could also add complexity. This is shown in Figure 2*E* in which the Ca^{2+} signal decays much slower from the neurite region marked 1 compared with region 2, and the level of Ca^{2+} remains higher at the end of the trace ($F/F_0 = 1.4$ compared with ~ 1.2). This particular region of the PD neuritic tree contained several sites of Ca^{2+} influx (the numerous bulbous structures shown in Fig. 2*B*), which all contribute to the Ca^{2+} kinetics observed in the neurite at region 1.

Large arborizations were occasionally found at the primary neurite close to the soma (Fig. 5*C*). Localization of calcium channels to these structures and a reduction of Ca^{2+} diffusion into the neurite is evident, as indicated by both differences in rise kinetics (data not shown) and the spatial dependence of the maximal fluorescence signal (F_{max}) along the structure and into the neurite (Fig. 5*C*).

Although voltage-activated fast Ca^{2+} accumulation was always restricted to the described varicosities, we found that only 30–50% of these investigated varicosities show voltage-dependent Ca^{2+} accumulation under our voltage-clamp conditions. Many

regions that appeared to have the appropriate morphology did not respond to voltage stimulation, although other varicosities within the same neuron, or even on the same neurite, would (see Discussion).

Effective diffusion coefficient of Ca^{2+} in the neuropil estimated from the delay of Ca^{2+} signal rise

In regions in which only a single arborization or a very closely spaced group of arborizations was found, the *in vivo* diffusion coefficient of free calcium could be estimated. The following assumptions were made. (1) The measured Ca^{2+} influx occurs only from a single region small enough to be considered a point source from several micrometers away. (2) The diffusion of Calcium Green-1 (both calcium-bound and -unbound) is too slow to account for the observed kinetics of fluorescence change. (3) A Fickian 1-D diffusion model applies: $D_{\text{eff}} = \langle r^2 \rangle / 2t$, where D_{eff} is the effective diffusion coefficient for Ca^{2+} , and r is the mean distance traveled in time t . We also assume that the delay time, as defined in Materials and Methods, provides a reasonable estimate of the time (t) in the above equation. Assumption 1 has been demonstrated in the previous section; there are clearly isolated, discrete regions in which Ca^{2+} influx occurs from a small area. We validated assumption 2 by measuring the diffusion of the dye using multiphoton fluorescence photobleaching recovery (Brown et al., 1999). Using this technique, it is possible to measure the diffusion coefficient of the dye in thick samples *in vivo*. In eight independent measurements in various regions of the neuron, the diffusion coefficient for Calcium Green-1 was on the order of 1–5 $\mu\text{m}^2/\text{sec}$, assuming Fickian diffusion and a single diffusion coefficient model. Slow diffusion of Calcium Green-1 was expected in this system based on the relatively long time required for dye equilibration after iontophoresis. Based on these measurements of dye diffusion and the much more rapid time scales observed for spreading of the Ca^{2+} signal, we can neglect the diffusion of Ca-bound dye and assume that the observed delay is attributable entirely to Ca^{2+} movement along the neurite.

The calculated values of D_{eff} for Ca^{2+} 2–10 μm from the presumed Ca^{2+} influx point average $237 \pm 24 \mu\text{m}^2/\text{sec}$ ($n = 6$). This is similar to values reported by others for Ca^{2+} in cytosol (Allbritton et al., 1992; Gabso et al., 1997). Calculation of diffusion coefficients from data taken further away from the point of influx ($>10 \mu\text{m}$) yield larger values of D_{eff} . The most likely reason for this is that the simple Fickian diffusion model is too simple to explain the complex dynamics for Ca^{2+} diffusion in a real neuron. It is unlikely that D_{eff} is a constant in the cytoplasm; rather, it can vary with the strength of fixed and mobile buffering capacity, distance from nearby sites of Ca^{2+} entry, etc. (W. Zipfel and P. Kloppenborg, unpublished observations).

Effect of dopamine on Ca^{2+} signals

DA has two, potentially separate, effects on the PD neuron: (1) it decreases the excitability of the PD neuron by increasing two K^+ currents, I_A and $I_{O(\text{Ca})}$ (Kloppenborg et al., 1999); and (2) it greatly reduces and sometimes abolishes synaptic transmission from PD output synapses (Johnson and Harris-Warrick, 1990). Whereas the modulation of K^+ currents may contribute to reducing release, Ca^{2+} currents could be selectively modulated at synaptic nerve terminals in a way that is not easily detectable by voltage clamp from the soma (Johnson et al., 1999). To test this hypothesis, we determined whether DA could modulate the voltage-dependent neuritic Ca^{2+} accumulation. We first identified active regions in which rapid Ca^{2+} accumulation occurred

Figure 7. Dopamine effect on peak Ca^{2+} accumulation. *A* is an image of the region in the STG neuropil from which the data were obtained. *B–D* demonstrate a reduction of Ca^{2+} accumulation during bath application of 10^{-4} M dopamine. The red bar in *A* indicates the area of the line scan that was analyzed in *B–D*. *C* shows three representative line scan images before, during, and after application of DA (10^{-4} M). *D*, Extracted fluorescence data and fit lines from the line scans of *C* (for details, see Fig. 2). *B* shows the time course of dopamine-induced reduction of peak Ca^{2+} accumulation. Each point represents the maximal value of F/F_0 (determined from the fit line) during a 200 msec voltage pulse as shown in *C* and *D*. The gray rectangle indicates the time of DA superfusion. *E*, A region in which voltage-activated Ca^{2+} influx could be reproducibly measured for a long period (>1 hr) but which showed no statistically significant response to dopamine as shown in *F*. *F*, Time course of peak Ca^{2+} accumulation from the region shown in *E*. Data are plotted as in *B*.

and made sure that this voltage-activated Ca^{2+} response was stable over time by applying voltage pulses (of 1 per min) for 5 min. If the response was reproducible over this period of time, we found that the signal amplitude would remain stable for long periods (>1 hr); such regions were then used for studying the effects of 10^{-4} M bath-applied DA. The principal effect of DA was a reversible decrease in magnitude of the Ca^{2+} signal. An example is shown in Figure 7*A–D* in which the voltage-induced Ca^{2+} accumulation is shown before, during, and after DA application. The effect was fully developed after ~ 10 min of DA application. The Ca^{2+} signals returned slowly to control levels upon return to

normal saline, and full recovery was typically observed after ~ 30 min of wash.

To quantify the effects of DA on the voltage-evoked Ca^{2+} signals, we analyzed the peak amplitude, rise time, and decay time of the signal. In 4 of 11 experiments (neurons), we found statistically significant and reversible reductions in Ca^{2+} accumulation ($p < 0.025$, t test). In these varicosities that had a dopamine response, DA decreased the average peak amplitude of Ca^{2+} accumulation by $16.5 \pm 8\%$ [decreases were 22% ($p < 0.00002$), 22% ($p < 0.0000003$), 18% ($p < 0.00008$), and 4% ($p < 0.002$)]. No statistically significant change in the average time constant for

rise and decay was observed. In three of the remaining experiments, DA decreased the voltage-activated Ca^{2+} accumulation, but the effect did not reverse. In three experiments, there was no significant change in the voltage-induced Ca^{2+} accumulation during DA application (Fig. 7F). Finally, in one neuron in which we measured two different sites sequentially, Ca^{2+} accumulation was significantly decreased by DA at one site and unaltered at the other. Based on these results, we postulate that there is a spatially differential effect of DA on the varicosities within a single PD neuron; that is, DA modulates Ca^{2+} entry at certain sites, although others are not affected.

During the DA experiments, we also monitored the cell input resistance with small hyperpolarizing (10 mV) voltage steps or ramps. Under our experimental conditions in which most conductances other than Ca^{2+} were blocked, DA had no measurable effect on the cell input resistance (data not shown). This suggests that the DA effect on Ca^{2+} accumulation was not attributable to a simple increase in electrotonic decay with consequent loss of voltage control of the neurites.

DISCUSSION

The primary goal of this study was to explore the spatial distribution of voltage-activated Ca^{2+} accumulation in PD neurons and its modulation by DA within the intact stomatogastric ganglion. Multiphoton microscopy was used to explore Ca^{2+} accumulation with high spatiotemporal resolution deep in the neuropil under controlled voltage conditions. To study Ca^{2+} accumulation in isolation and to improve our voltage control of distal neurites (by making the neuron electrotonically more compact), we blocked other conductances with extracellular TEA, 4-AP, TTX, and cesium.

MPM is particularly well suited for Ca^{2+} imaging of fine neurites located deep in the intact nervous system. For multiphoton excitation, pulsed lasers ($\sim 10^{-13}$ sec pulse width at 80 MHz repetition rate) in the infrared are used, which have high enough peak powers to allow for the simultaneous absorption of two photons in the focal plane of the objective lens. The sum of the energies of the two lower energy photons is equivalent to that required for excitation in the visible wavelength range, and the emission is identical to that arising from a single photon excitation. Multiphoton infrared excitation is advantageous for many reasons. Long wavelength light penetrates more deeply into tissue because of lower intrinsic one-photon absorbance and scattering. The excitation is limited to the focal plane, resulting in reduced photodamage while providing optical sectioning equivalent to a confocal microscope. Spatially localized excitation (and therefore emission) allows for highly efficient collection without a detector pinhole, resulting in minimal loss of scattered fluorescence. Signals can be detected from single events without averaging, and, using the line scan mode (in which data are collected along a single line across the visual field), acquisition rates of 500 Hz or greater are possible.

We found that peak Ca^{2+} accumulation is highly localized, with voltage-activated Ca^{2+} accumulation primarily restricted to small, specialized compartments of the PD neuron. These compartments are characterized by no observable delay between onset of the voltage pulse and rise in $[\text{Ca}^{2+}]$. The fast rise in $[\text{Ca}^{2+}]$ could be fitted with a single time constant and the signal could be blocked by Cd^{2+} or 0 Ca^{2+} saline, suggesting that the location of fast Ca^{2+} accumulation reflects the distribution of voltage-activated Ca^{2+} channels. At this point, however, we cannot exclude the possibility that a secondary Ca^{2+} -activated Ca^{2+}

release from internal stores may also contribute to the Ca^{2+} accumulation. Flanking regions around these “active” sites have visible delays, lower maximal $[\text{Ca}^{2+}]$, and longer decay times of the $[\text{Ca}^{2+}]$ after the voltage pulse, suggesting that the Ca^{2+} that enters into the active compartments can diffuse from there into other parts of the cell. Regions in which maximal rapid voltage-activated Ca^{2+} accumulation occurs are morphologically distinct varicosities on the neurites. Similar varicosities have been described by King (1976a) using transmission electron microscopy. King found that these varicosities are morphologically specialized regions in which many synaptic sites are located. Although presynaptic and postsynaptic sites could be found on the same neuritic branch, a single varicosity usually contained only presynaptic or postsynaptic sites.

A tempting interpretation of our data are that the sites of fast voltage-activated Ca^{2+} accumulation are the varicosities containing presynaptic membrane specializations seen by King (1976a); varicosities that did not show voltage-dependent Ca^{2+} accumulation could be postsynaptic sites. The structural specialization of these varicosities has obviously important physiological consequences. The increase in surface area provides the structural basis to place several synapses close to each other on the neurites that are then exposed to a similar chemical environment of the varicosity. Compared with the diameter of the varicosities, the diameter of the neurite is small. Thus, the varicosity forms a microdomain in which high $[\text{Ca}^{2+}]$ is selectively reached during a membrane depolarization (Fig. 5C). The larger volume of the varicosities argues against the possibility that the relatively higher Ca^{2+} concentration at these sites is attributable to differences in surface-to-volume ratio, because this would have yielded the opposite result.

Localized sites of calcium entry are also found in a number of other systems. In both vertebrates and invertebrates, calcium imaging studies have demonstrated that localized high densities of voltage-activated channels are present at presynaptic sites (Robitaille et al., 1990; Delaney et al., 1991; Eliot et al., 1993; Smith et al., 1993; Schweizer et al., 1995; Zucker, 1996; Karunanithi et al., 1997), and voltage- and ligand-activated channels with high Ca^{2+} conductance are localized at postsynaptic sites along dendrites and dendritic spines (Christie et al., 1995; Magee et al., 1998; Takechi et al., 1998; Cochilla and Alford, 1999; Yin et al., 1999). Localized increases in Ca^{2+} can play an important role in synaptic plasticity, such as long-term potentiation and long-term depression (Yang et al., 1999; Zucker, 1999).

We found that DA markedly reduces the Ca^{2+} accumulation at some, but not all, of the investigated “hot spots.” Johnson and Harris-Warrick (1990) showed previously that DA greatly reduces or even eliminates transmission at PD output synapses. The simplest interpretation of these data are that DA directly modulates synaptic strength at least in part by reducing the activity of the voltage-activated Ca^{2+} currents in specialized synaptic varicosities. The decrease in synaptic strength could be directly caused by the reduction of the localized Ca^{2+} accumulation. Currently, we do not understand why DA does not equally affect all varicosities that show voltage-activated Ca^{2+} accumulation in the PD neuron; there was no detectable change in Ca^{2+} accumulation at 3 of 11 experiments we studied (Fig. 7E,F). We have tested the outputs from the PD neurons onto all of their follower neurons in the pyloric network (Johnson and Harris-Warrick, 1990; Johnson et al., 1995); all were reduced by DA. Thus, it is possible that the non-DA-sensitive varicosities are not presynaptic terminals onto these neurons. Clearly, further experiments

will be necessary to determine the physiological significance of this result.

There is strong evidence that dopamine-evoked reduction of release is caused by reducing calcium currents in goldfish gonadotrophs (Van Goor et al., 1998), bovine adrenal chromaffin cells (Bigornia et al., 1990), rat pituitary cells (Nussinovitch and Kleinhaus, 1992), and rat lactotroph cells (Lledo et al., 1990). However, it is not clear whether reduction of Ca^{2+} entry is the only mechanisms by which DA reduces release. Release shows a highly nonlinear dependence on intracellular Ca^{2+} (Lando and Zucker, 1994; Zucker, 1996), so a small reduction in intracellular Ca^{2+} could cause a significant reduction in release. Our measurements showed an average 16% reduction in $[\text{Ca}^{2+}]$ at DA-sensitive varicosities, but it should be remembered that we were measuring the bulk concentration of free Ca^{2+} averaged over the entire volume of the varicosities, whereas release responds to the very high local concentrations of Ca^{2+} around the mouth of the Ca^{2+} channel pore (Llinas et al., 1992, 1995). Dopamine has also been shown to reduce the excitability of the PD neuron by increasing I_A and $I_{O(\text{Ca})}$ (Kloppenborg et al., 1999), and if these channels are localized at nerve terminals, they could also contribute to reducing release from PD neurons.

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