Heterogeneous Effects of Dopamine on Highly Localized, Voltage-Induced Ca\textsuperscript{2+} Accumulation in Identified Motoneurons

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Kloppenburg P, Zipfel WR, Webb WW, Harris-Warrick RM. Heterogeneous effects of dopamine on highly localized, voltage-induced Ca\textsuperscript{2+} accumulation in identified motoneurons. J Neurophysiol 98: 2910–2917, 2007. First published August 29, 2007; doi:10.1152/jn.00660.2007. Modulation of synaptic transmission is a major mechanism for the functional reconfiguration of neuronal circuits. Neurotransmitter release and, consequently, synaptic strength are regulated by intracellular Ca\textsuperscript{2+} levels in presynaptic terminals. In identified neurons of the lobster pyloric network, we studied localized, voltage-induced Ca\textsuperscript{2+} accumulation and its modulation in varicosities on distal neuritic arborizations, which have previously been shown to be sites of synaptic contacts. We previously demonstrated that dopamine (DA) weakens synaptic output from the pyloric dilator (PD) neuron and strengthens synaptic output from the lateral pyloric (LP) and pyloric constrictor (PY) neurons. Here we show that DA modifies voltage-activated Ca\textsuperscript{2+} accumulation in many varicosities in ways that are consistent with DA’s effects on synaptic transmission: DA elevates Ca\textsuperscript{2+} accumulation in LP and PY varicosities and reduces Ca\textsuperscript{2+} accumulation in PD varicosities. However, in all three neuron types, we also found varicosities that were unaffected by DA. In the PD neurons, we found that DA can simultaneously increase and decrease voltage-evoked Ca\textsuperscript{2+} accumulation at different varicosities, even within the same neuron. These results suggest that regulation of Ca\textsuperscript{2+} entry is a common mechanism to regulate synaptic strength in the pyloric network. However, voltage-evoked local Ca\textsuperscript{2+} accumulation can be differentially modulated to control Ca\textsuperscript{2+}-dependent processes in functionally separate varicosities of a single neuron.

INTRODUCTION

In neurons, Ca\textsuperscript{2+} elevation is the essential trigger for synaptic transmitter release and plays an important role as a second messenger for cellular processes such as membrane excitability, enzyme activation, and activity-dependent gene activation (Augustine et al. 2003). Cytosolic Ca\textsuperscript{2+} levels are not uniform, but can be compartmentalized and differentially controlled. One parameter that contributes significantly to the spatial and temporal dynamics of intracellular free calcium is the spatial distribution of voltage-activated Ca\textsuperscript{2+} channels, which can vary significantly in functionally specialized subcellular regions such as the soma, dendrites, dendritic spines, and presynaptic terminals.

In synaptic terminals, high levels of free intracellular Ca\textsuperscript{2+} induce transmitter release. Direct or indirect modulation of localized presynaptic Ca\textsuperscript{2+} influx is one important intracellular pathway to alter synaptic strength (Hille 2001; Reid et al. 2003; Schneggenburger and Neher 2005). To study the modulation of localized voltage-dependent Ca\textsuperscript{2+} accumulation, we use the pyloric network in the crustacean (Panulirus interruptus) stomatogastric ganglion (STG; Harris-Warrick et al. 1992). This network is a small, defined central pattern generator network that controls food processing; it has served as an excellent model for neuromodulation of neuronal networks at the cellular and synaptic levels (Harris-Warrick et al. 1998; Marder and Bucher 2007; Marder and Thirumalai 2002).

The pyloric circuit consists of 14 identifiable neurons, with known synaptic connections. The two pyloric dilator (PD) neurons, along with the anterior burster (AB) neuron, form the pacemaker group that sets the cycle frequency (Ayali and Harris-Warrick 1999). All other neurons, including the lateral pyloric (LP) and the eight pyloric constrictor (PY) neurons, are follower neurons. The pyloric neurons are connected by graded and spike-evoked inhibitory (cholinergic, glutamatergic) synapses as well as both rectifying and nonrectifying electrical synapses (Hartline and Graubard 1992; Johnson et al. 1993).

Dopamine (DA) functionally reconfigures the pyloric network (J) by distributed effects on ionic currents that shape the neurons’ intrinsic properties and 2) by modulating synaptic strength throughout the network. Among other actions, DA enhances the synaptic output of the LP and PY neurons and decreases the synaptic output from the PD neurons (Johnson and Harris-Warrick 1990, 1997; Johnson et al. 1995, 2005). Soma voltage-clamp studies suggest that DA acts by increasing (LP, PY) or decreasing (PD) voltage-activated Ca\textsuperscript{2+} currents (Johnson et al. 2003). We performed a calcium imaging study (Kloppenburg et al. 2000), showing that high-voltage-activated Ca\textsuperscript{2+} influx in PD neurons is highly localized to spatially restricted varicosities that were previously shown to be synaptic sites (King 1976a). In many of these varicosities, Ca\textsuperscript{2+} influx was reduced by DA, suggesting DA reduces chemical transmission from the PD neurons at least in part by decreasing Ca\textsuperscript{2+} entry at neurotransmitter release sites. However, DA had no effect at other PD varicosities, suggesting that Ca\textsuperscript{2+} influx is not modulated homogeneously throughout a single neuron.

The purpose of this study was to extend these results to different classes of pyloric neurons, to see whether modulation of localized Ca\textsuperscript{2+} influx is a common mechanism to regulate synaptic strength. We also analyzed DA modulation of Ca\textsuperscript{2+} entry at multiple varicosities in a single neuron, to determine...
whether localized Ca\(^{2+}\) entry is differentially modulated at different sites in a single neuron.

**METHODS**

Spiny lobsters, *Panulirus interruptus*, were obtained from commercial sources and maintained ≤4 wk in artificial seawater at 16°C until use. Calcium Green-1 was obtained from Molecular Probes (Eugene, OR). Other chemicals were obtained from Sigma Chemical (St. Louis, MO).

**Preparation**

Animals were anesthetized by cooling in ice for ≥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\(^{-1}\)) with saline (16°C) of the following composition (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl\(_2\), 3.9 Na\(_2\)SO\(_4\), 10.0 MgSO\(_4\), 2 glucose, 11.1 Tris base, pH 7.35 (Mulloney and Selverston 1974). Extracellular recordings were made from identified motor nerves using bipolar pin electrodes insulated by Vaseline (Kloppenburg et al. 2000). After desheathing the STG, individual somata were impaled with glass microelectrodes (10–25 MΩ; 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 preparation in its recording chamber was transferred from the identification rig to the imaging setup. The recording chamber was mounted on the modified temperature-controlled stage of a modified Olympus AX-70 upright microscope (Olympus, Melville, NY). The preparation was constantly superfused with saline (3 ml min\(^{-1}\)) at 16°C.

Detailed procedures for dye loading and estimation of intracellular dye concentration were described previously (Kloppenburg et al. 2000). Briefly, Calcium Green-1 (2 mM in H\(_2\)O) was loaded iontophoretically into single neurons using hyperpolarizing current. The injection was standardized to a −10-NA hyperpolarizing current for 20 min, when in all neuron types fine neuritic arborizations were visible. Using these parameters the injected dye had no immediate effect on the firing properties of the neuron, which was monitored in a rhythmically active preparation during brief interruptions of the current injection. From the amount of dye injected and the cell volume we estimated a total concentration of about 150–200 μM Calcium Green-1. Using ratiometric measurements with Indo-1 we previously determined the resting and maximal levels of free intracellular Ca\(^{2+}\) and demonstrated that this does not saturate Calcium Green-1 (Kloppenburg et al. 2000). Using a resting level of about 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\(_g\) of 190 nM, the peak [Ca\(^{2+}\)] reached during a voltage pulse to 0 mV was about 400 nM. The in vivo K\(_g\) of Calcium Green-1 in this system is not known and is a potential source of error in this estimate. All measurements are given as qualitative ratios of calcium signal relative to the baseline level at the holding potential of −45 mV. Imaging was not started until approximately 1 h after loading to allow for a uniform distribution of dye throughout the neuron and time for bath-applied blockers to take effect.

To remove chemical synaptic input to the dye-loaded neurons, 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 conductances other 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). Tetraethylammonium (TEA, 2 × 10\(^{-2}\) M) was used to block I\(_{K_{ATP}}\) and I\(_{K_{Ca}}\). I\(_{A}\) was eliminated by holding the PD neuron at −45 mV, where I\(_{A}\) is almost completely inactivated (Baro et al. 1997; Kloppenburg et al. 1999), and/or by 4 × 10\(^{-3}\) M 4-aminopyridine (4AP). In the PD neuron 4AP initially induced a depolarizing leak current (Kloppenburg et al. 2000) that disappeared within 30 min in the continued presence of 4AP (BR Johnson and P Kloppenburg, unpublished observation). To compensate for changes in osmolarity, the NaCl concentration was reduced.

**Voltage clamp of synaptically isolated neurons**

Synaptically isolated neurons were impaled with two electrodes for voltage recording and current injection (10 MΩ; 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 PC.

**Dopamine application**

Dopamine (DA, 10\(^{-4}\) M) was bath-applied at 3 ml min\(^{-1}\) 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 1986b).

**Imaging**

The combined multiphoton microscope/electrophysiology setup consisted of a Spectra Physics Tsunami Ti:S laser pumped by a 10-W Millennia solid-state (DPSS) 532-nm laser (Spectra Physics, Mountain View, CA), a modified BioRad MRC-600 scan-box, and a custom-built, fixed-stage Olympus AX-70 upright microscope. A Hamamatsu HC125-02 photomultiplier (Hamamatsu, Bridgewater, NJ) tube placed directly above the objective lens was used to collect the nondescanned emission (500–600 nm). The beam intensity was controlled using a ConOptics Model 350-50 Pockels Cell (ConOptics, Danbury, CT), which also blanked the laser during fly-back (in between scan lines), eliminating unnecessary excitation of the preparation.

Neurons loaded with Calcium Green-1 were imaged with 800-nm excitation (~100 fs before the microscope optics) through an Olympus ×200/0.5 NA or ×40/0.8 NA water-immersion objective lens. Calcium transients were acquired using line scans at a rate of 2 or 4 ms per line. Voltage-clamp data were simultaneously recorded on the second channel of the BioRad scanner during the line scans to synchronize the start of the voltage pulse with the Ca\(^{2+}\) signal.

**Data analysis**

Data extraction was performed by laboratory-written software. Pixel values were extracted from the line scan images along the time axis in the area of interest (averaged across the spatial axis). The simultaneously acquired membrane potential was used to determine the start and endpoints of the voltage pulse, ensuring synchronization between the Ca\(^{2+}\) data and the voltage pulse. The rise of the Ca\(^{2+}\) signal during the 200-ms voltage pulse was fit with

\[
F = \frac{A}{1 + \frac{e^{t_{0.5}\text{m}}}}
\]

where the amplitude A = (F\(_{\text{max}}\) − 1), t\(_{0.5}\) is the time when the half-maximal rise in fluorescence is reached, and s is a slope factor. The subsequent decay was fit with
\[ \frac{F}{F_0} = 1 + A \times e^{-t/\tau} \]

where \( \tau \) is the time constant for decay.

**Statistical analysis**

For a particular experiment, the DA-induced change in Ca\(^{2+} \) signal was considered significant if the change in the peak amplitude was >3 SD (\( \sigma \)). 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**

**Sites of Ca\(^{2+} \) measurement**

The three-dimensional (3D) structure of the Calcium Green-1–loaded PD neuron was visualized with high spatial resolution in the living ganglion. Multiphoton Ca\(^{2+} \) measurements were performed with repeated line scans (250–500 Hz) of enlarged varicosities that are localized on finer processes within the neuropil. We evoked Ca\(^{2+} \) accumulation in a voltage-clamped neuron with a 200-ms voltage pulse to 0 mV from a holding potential of −45 mV. We used a combination of ion channel blockers to reduce or eliminate noncalcium currents (Kloppenburg et al. 2000). This procedure sufficiently decreased the electronic decay to observe voltage dependence of the Ca\(^{2+} \) signal that paralleled that of \( I_{\text{Ca}} \) measured in voltage clamp from the soma (Johnson et al. 2003; Kloppenburg et al. 2000). Although the absolute voltage might differ somewhat from the measured voltage due to imperfect space clamp of the distal neuritic compartments, we assume reasonable voltage control of distal regions of the neuron. Voltage-activated fast Ca\(^{2+} \) accumulation was always restricted to the described varicosities. However, in all cell types, only 30 to 50% of varicosities investigated showed 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, even though other varicosities within the same neuron, or even on the same neurite, would.

**Effect of dopamine on Ca\(^{2+} \) signals**

First, we identified varicosities that showed rapid voltage-activated Ca\(^{2+} \) accumulation. When these responses were stable, we tested the effects of bath-applied DA (10\(^{-4} \) M). Although our earlier electrophysiological studies indicated that DA uniformly modulates synaptic output and somatic Ca\(^{2+} \) currents in a given type of pyloric neuron (Johnson and Harris-Warrick 1990, 1997; Johnson et al. 1995, 2005), the imaging results point to a more complex situation at the subcellular level (Kloppenburg et al. 2000). To quantify the effects of DA on the voltage-evoked Ca\(^{2+} \) signals, we analyzed their peak amplitudes, rise times, and decay times. In all three types of neurons (PD, LP, PY), a large number of varicosities showed a significant and a reversible change in the magnitude of the Ca\(^{2+} \) signal. These effects were fully developed after about 10 min of DA application. The DA-evoked changes in Ca\(^{2+} \) signals reversed, sometimes slowly, during washout to normal saline, and usually returned to the control values after \( \approx \)30 min. In all three cell types DA induced a significant change in the peak amplitude of the Ca\(^{2+} \) signal, with no reproducible effects on the time constant for rise and decay of the Ca\(^{2+} \) signal. During the DA experiments, we also monitored the membrane resistance with small (10-mV) hyperpolarizing voltage steps (Kloppenburg et al. 2000). Under our experimental conditions in which most conductances other than Ca\(^{2+} \) were blocked, DA had no measurable effect on the membrane resistance (data not shown). Together with the finding of increasing and decreasing Ca\(^{2+} \) influx at different locations this suggests that the DA effect on voltage-dependent Ca\(^{2+} \) accumulation is not due to a simple change in electroionic decay from the electrodes in the soma with consequent change of voltage control of the neurites.

**FIG. 1.** Pyloric dilator (PD) neuron. Dopamine (DA)-induced decrease of voltage-activated Ca\(^{2+} \) accumulation. A: neurite of a Calcium Green-1–loaded PD neuron from which the data were obtained (×400/0.8 NA). Position of the line scans is marked with a white line. Red line shows the area of the line scan that was analyzed in B–D. Scale: 10 \( \mu \)m. B–D demonstrate a reduction of voltage-induced Ca\(^{2+} \) accumulation during bath application of DA (10\(^{-4} \) M). B: time course of the DA-induced decrease of the peak Ca\(^{2+} \) accumulation. Each point represents the percentage change in the maximal value of \( F/F_0 \) during a 200-ms voltage pulse (as measured in D). C: line scans before, during, and after bath application of DA (10\(^{-4} \) M). Ca\(^{2+} \) accumulation was induced by a 200-ms voltage pulse from −45 to 0 mV (black bars). D: extracted fluorescence data and fit lines from the corresponding line scans in C.
slow unbinding of calcium from the dye. Dopamine caused a large reversible reduction in voltage-activated calcium signal in this example; this effect reversed after 20 min. However, in 23 varicosities (51%), there was no significant change in the voltage-induced \( \text{Ca}^{2+} \) accumulation during DA application (Fig. 2; original data not shown). Figure 2 shows the effects of DA on all 45 recordings. This figure demonstrates the clear separation between varicosities that respond reversibly to DA with a decrease in \( \text{Ca}^{2+} \) signal and those that do not respond to DA at all.

There are two PD neurons in each ganglion. These are thought to have very similar electrophysiological properties (Harris-Warrick et al. 1992; Schulz et al. 2006). However, the finding that only half of PD varicosities respond to DA leads to the hypothesis that only one of the PD neurons responds in this way to DA and the other does not. We considered this unlikely because the synaptic outputs of all PD neurons recorded were reduced by DA (Johnson and Harris-Warrick 1990). We were able to disprove this hypothesis using simultaneous recordings from two different varicosities within a single PD neuron; these recordings showed that the DA effect is not homogeneous throughout a single neuron. In five of nine PD neurons in which we simultaneously measured two different varicosities, we found that \( \text{Ca}^{2+} \) accumulation was significantly decreased by DA at one site but unaltered at the other varicosity. In the remaining four neurons, one responded with a decrease in both varicosities, and in three neurons both varicosities did not respond (data included in Fig. 2). These data are consistent with the average data over all cells that 50% of varicosities respond to DA and clearly show a differential effect of DA on the varicosities within a single PD neuron; that is, DA modulates \( \text{Ca}^{2+} \) entry at certain sites, whereas others are not affected.

LP NEURON. Dopamine enhances synaptic output from the LP neuron (Johnson et al. 1995, 2005). Accordingly, we predicted that DA would enhance voltage-dependent \( \text{Ca}^{2+} \) accumulation in LP varicosities. We investigated 39 varicosities in 28 LP neurons, including 11 neurons in which two sites were monitored simultaneously. In 18 varicosities (46%), DA significantly and reversibly enhanced the peak amplitude of the voltage-evoked \( \text{Ca}^{2+} \) signal, with a mean increase of 13.1 ± 4.6%. An example of such an experiment is shown in Fig. 3. However, in 21 (54%) varicosities there was no change in the voltage-induced \( \text{Ca}^{2+} \) accumulation during DA application. The distribution of values for all experiments is shown in Fig. 4; as with the PD neuron, there is a clear separation between the responding and the nonresponding varicosities, and not simply a Gaussian distribution of responses. Unlike the two PD neurons in each ganglion, there is only a single LP neuron, leading to two hypotheses to explain this heterogeneity of DA responses: either some LP neurons respond to DA with an increase in \( \text{Ca}^{2+} \) signal at all its varicosities whereas others do not, or there is heterogeneity in the individual varicosity responses within a single LP neuron. To test this, we simultaneously measured two different sites in a series of 11 LP neurons. In 5 neurons, we found that \( \text{Ca}^{2+} \) accumulation was significantly increased by DA at one site and unaltered at the other. Among the remaining 6 neurons, 2 responded with an increase in signal in both varicosities, whereas 4 showed no DA response in either varicosity. These data show that there is a spatially differential effect of DA on the varicosities within a single LP neuron; that is, DA modulates \( \text{Ca}^{2+} \) entry at certain sites, whereas others are not affected.

PY NEURON. Finally, we measured the effect of DA on voltage-activated \( \text{Ca}^{2+} \) accumulation in 89 varicosities from 72 PY neurons, including 17 experiments in which two varicosities were analyzed simultaneously from a single neuron. Because DA enhances synaptic output from PY neurons (Johnson and Harris-Warrick 1997), we expected the PY neuron varicosities to show a mixture of enhanced \( \text{Ca}^{2+} \) responses and no responses to DA, as we had found with the LP neuron. However, the results revealed an unexpected complexity in PY responses. In 34 (38%) of the PY varicosities, DA enhanced \( \text{Ca}^{2+} \) accumulation significantly and reversibly, as we predicted. The average increase in \( F/F_0 \) ratio was 14.4 ± 4.0%. As expected from our PD and LP results, a subpopulation of 37 varicosities (42%) did not change their voltage-induced \( \text{Ca}^{2+} \) accumulation during DA application. Unexpectedly, however, DA significantly and reversibly reduced the \( \text{Ca}^{2+} \) signal in the remaining 18 (20%) PY neuron varicosities, with a mean reduction in \( F/F_0 \) of 15.6 ± 4.5%. Figure 5 shows a summary of all 89 varicosities that were measured in 55 neurons, showing once again the clear separation between the three responses to DA.

Because there are eight PY neurons, these differential effects could reflect different responses to DA among the eight neurons, or different functional states of the neurons. Alternatively, different varicosities within a single neuron could respond differently to DA. To distinguish these possibilities, we performed 17 experiments in which two sites were imaged simultaneously in a single neuron during DA application. In six of these PY neurons, we found opposite effects of DA between

![Figure 2](image-url)
spatially opposite modulatory effect of dopamine within a neuron's intrinsic firing properties has been carefully studied (Flamm and Harris-Warrick 1986a,b; summarized in Harris-Warrick et al. 1998; Johnson and Harris-Warrick 1990, 1997; Johnson et al. 1994, 1995, 2005). Although the ionic mechanisms underlying the modulation of the neurons’ intrinsic firing properties has been carefully studied (Gruhn et al. 2005; Harris-Warrick et al. 1995a,b; Johnson et al. 2003; Kloppenburg et al. 1999, 2000; Peck et al. 2001, 2006), the mechanisms underlying DA modulation of synaptic strength are less well understood. Electrophysiologically, all the output synapses from the PY and LP neurons are enhanced, whereas the strengths of all the PD synapses are decreased (Johnson and Harris-Warrick 1990, 1997; Johnson et al. 1995). These and other studies suggest that DA can modulate transmitter release by a direct effect at the presynaptic terminal, which we verified with multiphoton microscopy calcium imaging studies here and in previous work (Kloppenburg et al. 2000).

Although there are many ways to modify presynaptic transmitter release (Katz 1999), many of them converge, either directly or indirectly, to modulate voltage-activated presynaptic Ca^{2+} currents. Our previous voltage-clamp studies of $I_{\text{Ca}}$
(Johnson et al. 2003) supported this hypothesis: in parallel with its physiological effects, DA enhances voltage-activated Ca\textsuperscript{2+} currents in the LP and PY neurons and decreases $I_{Ca}$ in the PD neuron. These electrophysiological experiments, however, were made from the soma and could not determine whether presynaptic Ca\textsuperscript{2+} influx is modulated.

To test this, we monitored the effects of DA on voltage-activated calcium accumulation in varicosities in the neuropil of the PD, LP, and PY neurons. The electron microscopic examination of the neuropil of STG neurons (King 1976a) showed that synaptic contacts are mainly located at such varicosities, and a single varicosity usually contained only presynaptic or postsynaptic sites. However, pre- and postsynaptic sites can be found on the same neurite. We have proposed that the varicosities that showed strong voltage-dependent calcium accumulation represent presynaptic terminals, whereas varicosities with no significant voltage-activated calcium accumulation represent postsynaptic terminals (Kloppenburg et al. 2000). Here we recorded from varicosities only with strong voltage-sensitive Ca\textsuperscript{2+} signals. Previously we could demonstrate a clear, reproducible voltage dependence of the Ca\textsuperscript{2+} signal (Kloppenburg et al. 2000). When voltage pulses of increasing amplitude were applied from a holding potential of $-45$ mV, Ca\textsuperscript{2+} accumulation can be detected starting at voltages more depolarized than $-40$ mV. From these data we cannot clearly differentiate whether the observed Ca\textsuperscript{2+} influx relates to graded or to spike-mediated transmission.

Our imaging studies show that DA modulates the Ca\textsuperscript{2+} signals in varicosities of PY, LP, and PD neurons in different ways. In each neuron, the sign of DA modulation of the Ca\textsuperscript{2+} accumulations in a significant fraction of varicosities is consistent with DA’s effect on synaptic transmission and the whole cell calcium currents in that neuron (Johnson and Harris-Warrick 1990, 1997; Johnson et al. 1994, 1995, 2003). DA elevates Ca\textsuperscript{2+} accumulation in synaptic varicosities of the PY and LP neurons, whose synapses are strengthened and $I_{Ca}$ is enhanced by DA. In contrast, DA reduces Ca\textsuperscript{2+} accumulation in varicosities of the PD neuron, whose synapses are weakened and $I_{Ca}$ is reduced by DA. These results support the straightforward hypothesis that DA alters synaptic strength at least in part by directly modulating localized synaptic Ca\textsuperscript{2+} influx.

However, the situation appears to be more complex than this. For all three neuron types, we found that DA does not have the same effects on all the varicosities that show voltage-activated Ca\textsuperscript{2+} accumulation, supporting our previous study of the PD neuron (Kloppenburg et al. 2000). In all three neurons, DA had no effect on voltage-activated Ca\textsuperscript{2+} accumulation in nearly half of the varicosities.

The PY neurons have even more complex responses than those of the other neurons to DA: in addition to varicosities that show increases or no change in voltage-activated Ca\textsuperscript{2+} accumulation, we found a significant fraction (20%) of varicosities that paradoxically showed a reduction in Ca\textsuperscript{2+} signal during DA application; this is opposite in sign to the effect of DA on the PY output synapses, which are uniformly enhanced by DA (Johnson et al. 2005). At first, we thought that these results could be explained by heterogeneity of the responses of different PY neurons to DA (Johnson et al. 2005): some but not
all neurons of a type would respond in a particular manner. However, this explanation is incorrect. We recorded the responses of two varicosities from single PY neurons during DA application and found that in all three neuron types, DA can have different effects on two varicosities within the same cell at the same time. These results suggest a strictly controlled differential modulation by DA within a single neuron. Consistent with our results, Clark and Baro (2006) reported that PD and LP neurons express different types of DA receptors (LP: D1aPan, PD: D2aPan). Further, in the PD neuron only a fraction of the varicosities contain the D2aPan receptor, whereas the remaining varicosities do not. This provides a possible molecular mechanism for the differential effects of DA on varicosities in the PD neuron. Future studies have to show whether selective localization of excitatory and inhibitory DA receptors in different varicosities of PY neurons can explain the tripartite responses of these neurons to DA.

On the cellular level, these findings fit the idea that microdomains with transient and sustained rises in cytoplasmic calcium concentration serve as second-messenger signals that control many cellular functions such as synaptic release, membrane excitability, enzyme activation, and activity-dependent gene activation. Selective triggering of these functions within specific microdomains but not others is achieved through spatial localization of calcium signals. The spatiotemporal distribution of calcium is determined by the geometric relationship between the Ca2+ source, Ca2+ buffering, and locally changing diffusion coefficients (Augustine et al. 2003).

At present, we do not understand the functional significance of this heterogeneity of DA responses at different varicosities within a single neuron. One possibility is that not all the varicosities are presynaptic terminals, despite their high-voltage–activated calcium accumulation. Future work labeling the terminals with synapse-specific markers after each experiment might be able to test this possibility. A second possibility could be that all the varicosities are presynaptic terminals, but DA differentially modifies those terminals onto specific postsynaptic targets. However, as stated earlier, the effects of DA are uniform on each neuron’s synaptic outputs. It is possible that these neurons synapse on other unknown targets; for example, they might form synapses onto axonal terminals of neurons descending to the STG from higher ganglia (Bartos and Nusbaum 1997; Coleman et al. 1995).

A third possibility is that the physiological strengthening or weakening of the synapse represents a summation of the differential effects of DA on the terminals from one neuron onto another. King (1976a,b) showed that, in P. interruptus, each neuron makes multiple contacts onto each of its postsynaptic target neurons. Thus if DA strengthens release from half of the LP terminals onto, for example, a PY neuron, with no effect on the others, the net effect will be a strengthened synapse. This could also explain the paradoxically opposite effects of DA on many of the PY varicosities: the net response would be a summation of the positive and negative modulations, with a net strengthening of the PY output synapses. One possible reason to limit the number of modified terminals, or to have terminals with opposing responses, is to limit the extent of modulation so that the synapse does not become too strong or too weak. A major problem facing strongly modulated networks like the pyloric network is how to maintain stability in output despite the multiple modulatory inputs, each of which has its specific actions on the network (Marder and Bucher 2007; Marder and Goaillard 2006). As we proposed previously (Harris-Warrick et al. 1998), one possible solution to this problem is for a modulatory input to exert at least partially opposing actions on a neuron. This would provide both a “drive” to change the pattern in a particular direction and a “brake” to limit that change to within the physiologically relevant range. In our earlier work, we showed that DA frequently has opposing actions on the pre- and postsynaptic sites of a synapse, for example, enhancing transmitter release presynaptically but reducing the postsynaptic responsiveness to that transmitter (Johnson and Harris-Warrick 1997). In addition, among the many modulatory effects of DA on voltage-dependent ionic currents that shape the intrinsic firing properties of pyloric neurons, we have found several examples of effects that appear opposed to the overall change in firing properties (Gruhn et al. 2005; Harris-Warrick et al. 1998; Peck et al. 2006). Our present results on differential and even opposing actions of DA on different varicosities in the same neuron provide further evidence for the hypothesis that a neuromodulator might self-limit its modulatory effects on a system by combining opposing or self-limiting effects on the system. This would retain the system within a physiologically relevant range and allow it to continue to function at all times.

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