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Dopamine-induced oscillations of the pyloric pacemaker neuron rely on release of calcium from intracellular stores

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Kadiril, Kwan AC, Webb WW, Harris-Warrick RM. Dopamine-induced oscillations of the pyloric pacemaker neuron rely on release of calcium from intracellular stores. J Neurophysiol 106: 1288–1298, 2011. First published June 15, 2011; doi:10.1152/jn.00456.2011.—Endogenously bursting neurons play central roles in many aspects of nervous system function, ranging from motor control to perception. The properties and bursting patterns generated by these neurons are subject to neuromodulation, which can alter cycle frequency and amplitude by modifying the properties of the neuron’s ionic currents. In the stomatogastric ganglion (STG) of the spiny lobster, Panulirus interruptus, the anterior burster (AB) neuron is a conditional oscillator in the presence of dopamine (DA) and other neuromodulators and serves as the pacemaker to drive rhythmic output from the pyloric network. We analyzed the mechanisms by which DA evokes bursting in the AB neuron. Previous work showed that DA-evoked bursting is critically dependent on external calcium (Harris-Warrick RM, Flamm RE. J Neurosci 7: 2113–2128, 1987). Using two-photon microscopy and calcium imaging, we show that DA evokes the release of calcium from intracellular stores well before the emergence of voltage oscillations. When this release from intracellular stores is blocked by antagonists of ryanodine or inositol triphosphate (IP3) receptor channels, DA fails to evoke AB bursting. We further demonstrate that DA enhances the calcium-activated inward current, ICAN, despite the fact that it significantly reduces voltage-activated calcium currents. This suggests that DA-induced release of calcium from intracellular stores activates ICAN, which provides a depolarizing ramp current that underlies endogenous bursting in the AB neuron.

RHYTHMIC ACTIVITY plays central roles in many aspects of nervous system function, ranging from motor control to perception. The neural mechanisms underlying rhythmogenesis are not well understood but include both network-driven oscillators and neuronal oscillators (Harris-Warrick 2010). Neuronal oscillators are single neurons that generate an endogenous oscillatory voltage pattern based on the ionic currents they express. The bursting properties of these neurons are subject to neuromodulation, which can alter cycle frequency and amplitude by modifying the properties of the neuron’s ionic currents (Harris-Warrick and Johnson 2010; Lotshaw et al. 1986; Marder and Bucher 2001; Marder et al. 2005; Partridge et al. 1990).

The pyloric network in the crustacean stomatogastric ganglion (STG) is an important system for studying the mechanisms of rhythmogenesis (Harris-Warrick 1993; Marder and Bucher 2001; Selverston and Ayers 2006). The pyloric circuit consists of 1 interneuron (the anterior burster, or AB) and 13 motoneurons. The AB neuron is a conditional oscillator: under the appropriate modulatory conditions, it generates intrinsic rhythmic oscillations and serves as the primary pacemaker to drive the network with a cycle frequency of 1–2 Hz. When isolated from all synaptic and modulatory input, the AB neuron loses its oscillatory capabilities. Addition of neuromodulators such as the monoamines dopamine (DA) and serotonin (5-HT) can restore rhythmic bursting in the isolated AB neuron. However, DA and 5-HT appear to use different sets of currents to drive the oscillations (Harris-Warrick and Flamm 1987). DA-induced bursting of the AB neuron is critically dependent on external calcium, as low external calcium or broad-spectrum calcium channel blockers halt it, while these blockers only slow the frequency of 5-HT-evoked bursting. In contrast, low external sodium concentration or application of tetrodotoxin (TTX) does not affect DA-induced oscillations but abolishes 5-HT-induced bursting (Harris-Warrick and Flamm 1987).

DA shapes the electrical properties of the AB neuron by affecting multiple currents in a cell-specific manner. DA reduces the tonic leak current, the transient potassium current (Peck et al. 2001), and the voltage-gated calcium current [ICa(V)] (Johnson et al. 2003). Additionally, DA enhances the hyperpolarization-activated inward current, Ih (Peck et al. 2006), which contributes to (but alone is not sufficient to trigger) the depolarizing ramp driving regenerative voltage oscillations. On the other hand, DA also enhances a slowly activating and deactivating inward-rectifying voltage-gated potassium current [IK(V)] (Gruhn et al. 2005), which may act to restrict the maximal spike frequency of the oscillating AB neuron (Harris-Warrick and Johnson 2010).

Here we have further studied the cellular mechanisms of DA-induced pacemaking in the AB neuron. The calcium dependence of DA-induced oscillations suggests an involvement of calcium currents and/or calcium-activated inward currents. However, DA reduces ICa(V) by up to 75% (Johnson et al. 2003), significantly reducing the influx of calcium ions into the cell. Since this does not appear to be the major source of calcium for DA-evoked bursting, we hypothesized that enhanced release of calcium from intracellular stores might be central to DA’s actions. In this paper, we show that DA does indeed evoke an accumulation of intracellular calcium by inositol triphosphate (IP3) receptor (IP3,R)- and ryanodine (Ry) receptor (RyR)-mediated release from intracellular stores. This increase in calcium concentration begins well before the emergence of
voltage oscillations, and in turn appears to activate the calcium-activated nonselective current I_{\text{CAN}} which provides a depolarizing ramp current that contributes to the pacemaker oscillations in the AB neuron.

MATERIALS AND METHODS

Animals and preparation. Adult California spiny lobsters (Panulirus interruptus) were supplied by Don Tomlinson Commercial Fishing (San Diego, CA) and kept in tanks with artificial seawater. Animal use followed a protocol approved by the Institutional Animal Care and Use Committee at Cornell University. Lobsters were anesthetized in ice; the stomatogastric nervous system (STNS) was dissected out, pinned on a Sylgard-coated petri dish, and superfused with oxygenated lobster saline at 16–17°C unless otherwise specified. The STG was desheathed, and cells were identified as previously described (Selverston et al. 1976). Experiments were primarily performed on the AB neuron, but some used the pyloric dilator (PD) and lateral pyloric (LP) neurons.

Solutions. Panulirus physiological saline had the following composition (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl2, 3.9 Na2SO4, 10.0 MgSO4, 2 glucose, and 11.1 Tris base, pH 7.4 (Mulloney and Selverston 1972). To pharmacologically isolate the ionic currents of the AB neuron, we used flufenamic acid (10 μM) and PTX (5 μM) to block the sodium current, and 5 μM picrotoxin (PTX) to block glutamatergic synapses within the STG; the NaCl concentration was adjusted to compensate for high TEA. In addition to the bath-applied blockers, we loaded the cell body with intracellular K+ channel blockers by iontophoresis, injecting small negative current steps through electrodes loaded with 2 M TEA and 2 M CsCl for 1–1.5 h; the cell was allowed to recover for 1–1.5 h before the experiment started. Although these treatments reduced K+ currents by >99%, a detectable outward current still contaminated our voltage-clamp studies of the very small calcium-dependent currents. We used flufenamic acid (FFA, 0.1–30 μM) to block I_{\text{CAN}}, 30 μM BAPTA-AM to chelate intracellular calcium ions, 10–100 nM Ry to block intracellular RyR channels, 1–10 μM xestospongin C or D to block intracellular IP3R channels, and 10 μM thapsigargin (Tg) to block the sarco/endoplasmic reticulum calcium-ATPase (SERCA) pump. FFA, Ry, xestospongin, and Tg were first dissolved in DMSO to make 100× stock solutions. Immediately before the experiment, the stock solution was diluted in saline to bring the concentration of the blockers to the desired level; the concentration of DMSO did not exceed 1% in the final solution. DMSO alone at this concentration did not have any significant effect on the physiology of the neuron. Chemicals were purchased from Sigma (BAPTA-AM, FFA, Ry), Tocris (Ry), or Cayman Chemical (xestospongin).

Two-photon calcium imaging. Calcium indicator dyes were injected into the AB neuron by iontophoresis (Kloppenburg et al. 2000). Only AB neurons that maintained rhythmic activity and showed labeling in fine neurites 30 min after iontophoresis were retained for analysis. We labeled the AB neuron with potassium salts of Calcium Green-1 (Don Tomlinson Commercial Fishes) and Calcium Orange-1 (Cayman Chemical). Calcium indicator dyes were in-jected before data analysis. For the determination of the I_{\text{CAN}} reversal potential, an activating prestep was followed by a series of hyperpolarizing steps from −80 mV to −40 mV in 5-mV increments to evoke a tail current. The peak slow tail current amplitude was measured and plotted against the value of the voltage step and extrapolated to determine the reversal potential (E_{\text{rev}}). The deactivating portion of the current trace was fitted with an exponential function using Clampex; the time constants were then plotted against voltage steps.

Data analysis. Electrophysiological data analysis was performed with Clampfit10 (Molecular Devices). The calcium imaging data were analyzed with ImageJ (National Institutes of Health). Synchronization of imaging and physiology was done with custom-written MATLAB software. Statistical analyses were performed with JMP 7 (SAS Systems). Graphs and figures were made in Excel, Origin 6 (OriginLab, Northampton, MA), and Adobe Illustrator CS3 (Adobe Systems). All values are presented as means ± SE; the effect was considered statistically significant at P < 0.05.
RESULTS

Release of calcium from intracellular stores is essential for DA-induced bursting in the AB neuron. In light of our previous results that DA induces calcium-dependent pacemaker oscillations (Harris-Warrick and Flamm 1987) while greatly inhibiting calcium influx via $\text{I}_{\text{Ca(V)}}$ (Johnson et al. 2003), we hypothesized that these oscillations rely on intracellular sources of calcium ions. DA could enhance $\text{Ca}^{2+}$ release from intracellular stores, inhibit $\text{Ca}^{2+}$ sequestration and buffering in intracellular compartments, or both.

We initially assessed the involvement of intracellular calcium stores in induction of pacemaker oscillations by chelating the intracellular calcium with lipid-permeable BAPTA-AM (Fig. 1A). This compound diffuses into the neuron, where it is deesterified to yield BAPTA. With time of diffusion the AB oscillations became smaller, and eventually the neuron ceased to oscillate and remained at a depolarized voltage. Similar results were seen by blocking the intracellular calcium-release channels located on the endoplasmic reticulum (ER) membrane such as Ry- and IP$_3$-sensitive intracellular calcium receptor channels, as well as SERCA, a calcium reuptake pump. Ry, a specific blocker of the intracellular calcium channels responsible for calcium-activated calcium release, blocked DA-induced AB neuron oscillations at concentrations of 10 nM or higher within 40 min ($n = 7$; Fig. 1B). Xestospongin C (Xe) is a specific blocker of the IP$_3$R channel (Gafni et al. 1997); 10 $\mu$M Xe effectively halted the DA-evoked AB rhythm ($n = 3$; Fig. 1C). Finally, application of 1 $\mu$M Tg, a specific blocker of the SERCA pump, stopped an ongoing DA oscillation within 40 min ($n = 3$; Fig. 1D). These data suggest that release of stored intracellular calcium via RyRs and IP$_3$Rs is essential for DA-induced oscillations in the AB neuron. Notably, as the DA-evoked bursting ceased with all of these blockers, the trough potential depolarized until the cell fell silent at a highly depolarized voltage (typically $-40$ to $-42$ mV). This was also observed when DA-evoked AB bursting was abolished by low extracellular calcium or calcium channel blockers (Harris-Warrick and Flamm 1987). This depolarization may be caused by an indirect block of calcium-sensitive potassium current [$I_{\text{KCa}}$] and subsequent depolarization of the $V_{\text{m}}$, as well as reflecting the effects of DA on other ionic currents such as inhibition of the leak conductance or activation of calcium-dependent inward currents (see below).

Calcium imaging reveals DA-evoked increase of intracellular $[\text{Ca}^{2+}]$ in AB neurites. To directly test the possibility that DA induces an increase in intracellular $\text{Ca}^{2+}$ levels, we carried out two-photon laser microscopic imaging of intracellular calcium activity in the AB neuron on two different timescales, using two different calcium-sensitive indicator dyes (Calcium Green-1 and Indo-1). Briefly, we loaded the AB neuron soma with the calcium indicator dye via the intracellular electrode, let the dye diffuse throughout the dendritic arbor for up to 1 h, and performed synchronized two-photon calcium imaging and electrophysiological recordings.

Distribution and strength of calcium signal in AB neuron. The dye-filled AB soma and neurites showed clear calcium fluorescence in both intact and DA-stimulated oscillating AB neurons. The signal from the cell body and the primary neurite was relatively constant, and we were unable to detect any oscillation-related fluorescence changes there. This is consistent with previous reports suggesting that calcium oscillations originate outside the soma in the fine neuropil (Kloppenburg et al. 2000, 2007; Ross and Graubard 1989). As a consequence, we made our measurements in small neurites with diameter <5 $\mu$m, at distances from the soma of >500 $\mu$m.

We performed timed ($6$–$10$ images/s) ratiometric measurements of Indo-1 fluorescence levels in fine neurites combined with simultaneous voltage recordings from the AB soma first in the absence and then in the presence of DA. Figure 2 shows an example of the AB soma and its extensive neuritic tree filled with Indo-1 (Fig. 2A, fluorescence from the 395-nm channel), where the yellow arrowheads indicate typical regions of neuropil used for calcium measurements and the dashed square demarcates the region enlarged in Fig. 2B. Within this region, the ratios of the fluorescence signals from the 395- and 495-nm channels were calculated and shown in Fig. 2B in pseudocolor. Additionally, the time course of the calcium signal from 19 regions of interest (ROIs) in neuropil was measured over the period of the whole experiment.

Fig. 1. Release of calcium from intracellular stores is essential for dopamine (DA)-induced anterior burster (AB) oscillations. A: 30 $\mu$M BAPTA-AM (BAPTA) blocked 10$^{-5}$ M DA-induced bursting in a synaptically isolated AB. In this experiment, the AB neuron was isolated from all synaptic and descending neurendiomial inputs by photoablation of the pyloric dilator (PD) and ventricular dilator (VD) neurons and local application of tetrodotoxin (TTX) to a Vaseline pool around the stomatogastric nerve. B–D: 10 nM ryanodine (Ry), 10 $\mu$M xestospongin C (Xe), and 1 $\mu$M thapsigargin (Tg) blocked DA-induced oscillations in the AB. In these experiments, the AB neuron was isolated by application of TTX and picrotoxin (PTX) directly to the stomatogastric ganglion (STG).
Basal level of intracellular calcium rises during DA application. Upon DA application, all visualized fine neurites displayed significant increases in calcium fluorescence of varying amplitude relative to their pre-DA levels (Fig. 2B and Fig. 3A). The calcium responses of 27 individual neurites from 3 different AB neurons were monitored as DA was applied to the bath. For individual ROIs in the fine neuropil, when averaged calcium measurements were taken at slow speed (6–10 images/s) the DA-induced increase in [Ca\(^{2+}\)]\(_{in}\) ranged from 136% to 555% of the pre-DA level with an average of 302 ± 153% increase. Thus different neurites of a single neuron showed marked variability in calcium handling both under control conditions and in the presence of DA.

Onset of basal [Ca\(^{2+}\)]\(_{in}\) rise precedes onset of voltage oscillations. The changes in [Ca\(^{2+}\)]\(_{in}\) were monitored in parallel with voltage recordings; for the large majority of neurites (25 of 27), the rise in [Ca\(^{2+}\)]\(_{in}\) started well before the emergence of sustained voltage oscillations (Fig. 3A). The intracellular calcium levels began rising ~3 min after DA reached the ganglion. The interval between the start of the calcium increase and the onset of voltage oscillations is highlighted with a vertical light gray bar in Fig. 3, A and B1. On average, voltage oscillations started 108 ± 12 s after the onset of calcium rise in fine neurites (n = 27 neurites, 3 AB neurons). This earlier onset of calcium rise in fine neurites, >1.5 min before the emergence of bursting, suggests that the rise in intracellular calcium might bring about the subsequent depolarization and rhythmic \(V_m\) oscillations. DA’s effect was completely reversible: washing DA out of the bath solution abolished the \(V_m\) oscillations and brought the levels of [Ca\(^{2+}\)]\(_{in}\) back to pre-DA levels (Fig. 3B). Figure 3B2 presents a summary of the mean intracellular [Ca\(^{2+}\)] under different conditions: in physiological saline, [Ca\(^{2+}\)] in a spontaneously oscillating AB neuron was 140 ± 8 nM. When AB neuron was synaptically isolated (either by photoablation of the electrically coupled PD and VD neurons or pharmacologically by bath application of TTX and PTX), the mean [Ca\(^{2+}\)] decreased to 67 ± 3 nM. Upon subsequent DA application the levels of calcium started rising within minutes; the voltage oscillatory activity usually started when the mean [Ca\(^{2+}\)]\(_{in}\) reached 135 ± 14 nM (not shown), while during stable DA oscillations time-averaged [Ca\(^{2+}\)] reached 193 ± 17 nM (for all conditions, n = 27). Calcium levels during DA application were significantly different from control measurements before DA in TTX or after washout (ANOVA, \(P < 0.01, n = 27\)).

Effect of preincubation with ryanodine and xestospongin on DA-evoked calcium rise. Our previous data on AB bursting predicted that application of the intracellular calcium channel blockers Ry and Xe would prevent or block the DA-induced increase in calcium levels and voltage oscillations. In three cells that were pretreated for 30–60 min with a cocktail of 10 \(\mu\)M Ry and 10 \(\mu\)M Xe, DA either elicited only a small calcium increase and a short-lived train of slow voltage oscillations or did not induce any significant increase in intracellular calcium and did not evoke voltage oscillations (Fig. 3C1). The mean [Ca\(^{2+}\)] was 82 ± 11 nM in Ry + Xe and 87 ± 17 nM in the presence of DA together with Ry + Xe, which were not statistically different (Fig. 3C2) (\(P > 0.05, n = 10\) ROIs).
Although DA failed to evoke sustained voltage oscillations in these neurons, it did slightly depolarize the $V_m$, probably because of its effect on other ionic currents in the AB neuron (Gruhn et al. 2005; Johnson et al. 2003; Peck et al. 2001, 2006).

High-temporal-resolution calcium imaging with simultaneous recording of membrane potential. We used Calcium Green-1 to track calcium changes on a faster timescale during spontaneous and DA-evoked AB oscillations. As noted above, we did not detect any calcium oscillations in the cell soma or primary neurites, but the fluorescence signals in fine neurites revealed clear oscillatory behavior at the same frequency as the $V_m$ oscillations.

To monitor calcium changes on a cycle-by-cycle basis, we used fast line scan (frequency 164 Hz) to monitor calcium activity in single fine neurites along with synchronized electrophysiological recordings of $V_m$ in the soma. In the spontaneously bursting AB (with intact modulatory input to the STG

![Figure 3. Onset of the DA-induced calcium rise precedes the onset of membrane potential ($V_m$) oscillations. A: time course of the voltage (top) and free Ca$^{2+}$ concentration ([Ca$^{2+}$]) bottom) change during DA application; 10$^{-4}$ M DA has reached the bath solution at the 50 s time point. Each dendrite [region of interest (ROI)] is indicated by different color; the data points from one of the neurites are fit with a sigmoid function (plotted as a red solid line). Note that in the time period highlighted with the vertical light gray bar, [Ca$^{2+}$] started rising before emergence of bursting. Voltage oscillations initially are slower and accelerate as the DA concentration reaches steady state. B1: complete time course of AB oscillations and intracellular calcium measurements as DA is added and washed out. As DA was washed out of the bath solution, the voltage oscillations and calcium signal returned back to their pre-DA levels within 10–15 min. B2: summary of the changes in the average calcium levels under different conditions: physiological saline (140 ± 8 nM), TTX (67 ± 3 nM), 10$^{-4}$ M DA (193 ± 17 nM), and washout of DA. The neuron was oscillating in saline and in the presence of DA and quiescent in TTX and after DA (wash). C: preincubation with intracellular calcium channel blockers (Ry+Xe) abolishes the DA-evoked rise in [Ca$^{2+}$], and oscillations in the AB neuron. C1: the AB neuron was preincubated with Ry+Xe for 30–60 min before DA application. Intracellular [Ca$^{2+}$] did not change upon DA application; the neuron depolarized, because of DA’s effects on other currents, but did not oscillate. C2: there was no significant difference in mean [Ca$^{2+}$] under different conditions: 82 ± 11 nM before DA (in Ry+Xe) and 87 ± 17 nM in the presence of DA and Ry+Xe ($P > 0.05, n = 10$ ROIs).
from the higher-order ganglia), [Ca$^{2+}$], monitored as the change in fluorescence level (dF/F), oscillated at the same frequency as the voltage (Fig. 4A). Application of TTX and PTX to block the descending modulatory inputs effectively blocked both voltage and calcium oscillations within several minutes (Fig. 4B). Addition of DA to the bath saline restored rhythm bursting of voltage along with calcium oscillations (Fig. 4C). Finally, Ry treatment of the bursting pacemaker in the continued presence of DA halted both calcium and voltage oscillations (Fig. 4D). The amplitude of the calcium oscillations was $\sim$20% of the basal level of calcium in the presence of DA. Notably, the calcium oscillations were slightly delayed relative to the voltage oscillations in both the spontaneously bursting AB neuron before isolation and the synaptically isolated AB treated with DA (Fig. 4E; $n=3$). The maxima of the calcium oscillations were delayed by $\sim$150 ms relative to the peaks of the $V_m$ oscillations. This delay seems to be too long to be explained by the slow kinetics of calcium activation of Calcium Green-1 (Parker and Yao 1996).

Dopamine-induced oscillations are blocked by FFA, a blocker of the $I_{\text{CAN}}$. Our imaging studies show that DA evokes increases in intracellular calcium that could lead to depolarizations preceding the onset of voltage oscillations. One possible target of the rise in $[\text{Ca}^{2+}]_{\text{in}}$ is the calcium-activated nonspecific current $I_{\text{CAN}}$. We tested the importance of $I_{\text{CAN}}$ in DA-evoked bursting by application of a blocker of this current, FFA. Three micromolar FFA reliably blocked the ongoing DA oscillations in a synaptically isolated AB neuron ($n=5$), regardless of the method used to synaptically isolate the AB (Fig. 5A). Notably, as the rhythm ceased, the trough potential depolarized until the cell fell silent at $-40$ to $-42$ mV. This was also observed when we blocked the rise in intracellular calcium (Fig. 1) and when DA-evoked AB bursting was abolished by low extracellular calcium or calcium channel blockers (Harris-Warrick and Flamm 1987). To rule out the possibility that this depolarization was responsible for the cessation of bursting, we injected a negative bias current (usually 1–5 nA) to bring the $V_m$ down to its preblocker trough values of $-55$ to $-60$ mV. Although early during FFA application AB bursting could be rescued by hyperpolarizing the $V_m$, perhaps after enough time to allow equilibration of the FFA concentration, the cell became completely unable to burst or oscillate, even when hyperpolarized. This lack of burst rescue by hyperpolarization suggests that it is the block of the FFA-sensitive inward current and not the possible secondary effect of depolarization that causes disruption of the DA rhythm. Therefore, we next focused on the characterization of the properties and DA modulation of $I_{\text{CAN}}$ in the pacemaker neuron.
Properties of $I_{CAN}$ in the anterior burster neuron. We have carried out a physiological and pharmacological analysis of $I_{CAN}$ by measuring its $E_{rev}$, deactivation time constant, and sensitivity to FFA. Because this current lacks intrinsic voltage sensitivity, and because of problems of space clamp in these highly branched neurons (Kloppenburg et al. 2000), we were unable to perform a full biophysical analysis. In the presence of blockers of ionic currents and synaptic input, we measured $I_{CAN}$ as a slowly deactivating tail current following a depolarizing prestep to $0$ mV to activate $I_{Ca(V)}$. The tail current was recorded over $-80$ mV to $-40$ mV in $5$-mV increments. Although we blocked $>99\%$ of the total K* currents, there was still some leak contamination of the very small calcium currents measured in the AB neuron (Fig. 5B). Figure 5B shows sample current traces without leak current subtraction. The peak amplitude of this tail current was measured at the beginning of the hyperpolarizing step, taking advantage of the much more rapid deactivation kinetics of the calcium current relative to that of $I_{CAN}$ in stomatogastric neurons (Johnson et al. 2003). At $-65$ mV, the mean amplitude of the peak leak-subtracted $I_{CAN}$ in the AB was $-2.7 \pm 0.7$ nA (Fig. 5E, open squares; $n = 8$). The leak-subtracted tail current was plotted as a function of the hyperpolarizing voltage step, fitted with a linear function, and $E_{rev}$ was extrapolated for each cell. The average $E_{rev}$ was $-29 \pm 0.3$ mV. The current decayed during the hyperpolarizing step, reflecting the time constant of channel deactivation and/or removal of intracellular calcium. The decay of the $I_{CAN}$ tail current was fitted with an exponential function, and resulting time constants $\tau$ were plotted against tail current voltage. We tested whether there was any voltage dependence of the deactivation rate. The current tended to decay somewhat more rapidly at voltages more depolarized than $-60$ mV, but there was no statistically significant difference between $\tau$ values at different voltages, with an average $\tau$ of $74 \pm 13$ ms.

As expected for $I_{CAN}$, the tail current was sensitive to application of FFA or BAPTA or block of calcium entry by CdCl$_2$. In the high-osmolarity lobster saline, FFA visibly precipitated out of solution at $16^\circ$C; thus we raised the experimental temperature to $21^\circ$C to maintain the drug in solution for full dose-response experiments. FFA blocked $I_{CAN}$ with an $IC_{50}$ of $24.7 \pm 5$ M; $n = 4$; Fig. 5C). Preventing a rise in intracellular calcium with $50-70$ M BAPTA completely blocked $I_{CAN}$. Finally, blockade of voltage-dependent calcium currents with $0.6$ mM CdCl$_2$ eliminated the step-activated $I_{Ca(V)}$ and thus the resulting $I_{CAN}$ (not shown).

We next measured DA’s effect on $I_{CAN}$ in the AB neuron. The mean peak amplitude of $I_{CAN}$ was increased in the presence of DA (Fig. 5D, thick black trace, arrowhead). For all eight neurons tested, this effect of DA was statistically significant at voltages more hyperpolarized than $-45$ mV ($P < 0.05$; Fig. 5E). This $I_{CAN}$ enhancement occurred despite DA’s marked reduction of the amplitude of $I_{Ca(V)}$ (by $68 \pm 7\%$; Fig. 5D, thick black trace, arrowhead; see also Johnson et al. 2003). The maximal CAN conductance in the AB neuron significantly
increased from 58 ± 9 nS under control conditions to 145 ± 12 nS in the presence of DA (248 ± 15% of control; n = 8, P < 0.05). We also tested DA’s effects on $I_{\text{CAN}}$ in two other pyloric network neurons, the PD, which is electrically coupled to the AB neuron, and the LP neuron. DA did not modify $I_{\text{CAN}}$ amplitude in the PD (105.8 ± 25% of control; n = 3) or the LP (103.1 ± 29% of control; n = 3) neurons.

The time course of decay of $I_{\text{CAN}}$ most likely reflects the reduction in availability of free calcium ions in the intracellular space near channels mediating $I_{\text{CAN}}$. Consistent with this, within each treatment (control, DA, and washout) there was no voltage dependence of the time constants of decay (ANOVA, $P > 0.05$, n = 7 for each treatment; not shown). However, when we compared the mean time constants between treatments (control and DA), the time constant in DA (150 ± 21 ms) was significantly slower compared with control (74 ± 14 ms) at −65 mV and more depolarized potentials ($P < 0.05$, n = 6). Thus, despite the fact that DA inhibits most of the $I_{\text{CAN}}$, the $I_{\text{CAN}}$ in DA is larger and deactivates more slowly than before DA application.

**DA effects on other ionic currents.** As reported previously (Peck et al. 2001), DA also reduced the leak current, measured with 5-mV hyperpolarizing steps from −60 mV or −55 mV. Under our experimental conditions, the mean conductance of the leak current at −5 mV was 45 ± 17 pS in control, 11 ± 8 pS during DA application, and 41 ± 23 pS after DA washout. This ~70% reduction in leak current in the presence of DA is larger than the 10% reduction we previously reported in the AB (Peck et al. 2001, 2006).

**DISCUSSION**

Our results suggest that DA-induced AB bursting depends on the release of calcium from intracellular stores, which in turn enhances $I_{\text{CAN}}$ to provide the additional ramp depolarization leading to the onset of each oscillation. Blockade of either of these steps eliminates or prevents pacemaker oscillations.

**Dopamine-induced rise in intracellular calcium is central for pacemaker oscillations.** Many bursting neurons rely on intracellular calcium stores as a source of calcium in both invertebrates (Levy 1992; Partridge and Swandulla 1987; Partridge and Valenzuela 1999; Sawada et al. 1990; Swandulla and Lux 1985; Yazenian and Byerly 1989) and vertebrates (Del Negro et al. 2008; Pace et al. 2007a, 2007b; Peña et al. 2004; Rubin et al. 2009). We show that in the pyloric pacemaker AB neuron chelation of calcium ions or block of calcium release from the intracellular stores prevents or eliminates ongoing DA-evoked pacemaker oscillations. Calcium imaging of the AB neuron with a ratiometric dye shows that DA elevates basal calcium levels in the neurites by more than twofold over the quiescent state (67 ± 3 nM pre-DA, 193 ± 17 nM in DA). We are able to detect this DA-induced elevation of [Ca$^{2+}$]$_{\text{in}}$ beginning more than a minute and a half before the emergence of sustained voltage oscillations (Fig. 3A) and, in some cells, even before the neuron starts to depolarize.

During AB oscillations, fast line scanning of neurites filled with Calcium Green-1 shows that the intracellular calcium level oscillates with amplitude of ~20% of the basal level of calcium in the presence of DA and with the same frequency as the voltage oscillations. However, the calcium oscillations are delayed from the voltage oscillations by ~150 ms (0.08 of the period). One interpretation of this result is that a tonic modulator-evoked release of calcium from intracellular stores activates $I_{\text{CAN}}$, which acts as a major contributor to the tonic ramp current, depolarizing the membrane voltage; above a voltage threshold, this depolarization activates the small DA-insensitive portion of $I_{\text{Ca(V)}}$, which then participates (possibly along with $I_{\text{CAN}}$) in the rapid rise of the oscillation to its peak. The essential role of calcium release from intracellular stores is maintained even during stable oscillations, since Ry blockade of calcium-induced calcium release abolishes these ongoing calcium oscillations along with voltage oscillations (Fig. 4D).

**Cross talk between IP$_{3}$ and Ry receptors and their respective pathways.** The ER calcium stores appear to play a critical role in DA-induced AB bursting, since blockade of Ry-sensitive and IP$_{3}$ receptors or depletion of stores by blockade of the SERCA inhibits this bursting. The mechanisms by which DA enhances calcium release from these stores are not yet known.

Clark and colleagues cloned three types of DA receptors from the lobster STG: $D_{1}\text{Pan}$ coupled with $G_{i}$ and $G_{s}$, $D_{1}\text{LP}$ coupled with $G_{i}$, and $D_{2}\text{LP}$ coupled with $G_{i}$ proteins (Clark and Baro 2006, 2007; Clark et al. 2008). All three receptor types were reported to localize to the synaptic neuropil (Clark et al. 2008), where we made our calcium measurements. In vertebrates, DA can trigger mobilization of intracellular calcium through concurrent activation of $D_{1}$ and $D_{2}$ receptors or a $D_{1}-D_{2}$ heteromer, via activation of $G_{i}$ phospholipase C (PLC), and the IP$_{3}$ cascade (Hasbi et al. 2010; Undie et al. 1994), which in turn provides calcium to activate further calcium release via the RyRs. In *Drosophila* photoreceptors, this signaling cascade leads to calcium release from IP$_{3}$Rs and calcium influx across the cell membrane via two types of TRP channels (Hardie and Minke 1993). Thus, in the AB neuron, DA most likely acts on the $D_{1}\text{Pan}$ receptor or its heteromer to activate the $G_{i}$-PLC-IP$_{3}$ pathway.

In addition, DA could also activate $D_{1}\text{LP}$ or $D_{1}\text{LP}$, which are coupled to the $G_{i}$-CAMP-PKA pathway (Clark et al. 2008), leading to increased phosphorylation of RyR protein and calcium release from the ER, as demonstrated in heart sinoatrial node pacemaker cells (Vinogradova et al. 2006). Since both types of ER receptor channels are sensitive to calcium, they are subject to positive autofeedback, leading to regenerative opening of RyRs and/or IP$_{3}$Rs beyond a certain threshold of [Ca$^{2+}$]$_{\text{in}}$ and amplification of the initial calcium signal (Verkhratsky 2005). Replenishment of the intracellular calcium stores is critically important to prolonged DA bursting; this may occur via $I_{\text{CAN}}$, $I_{\text{Ca(V)}}$, and/or store-operated calcium entry (SOCE). Although we did not test this directly in the AB neuron, in rat hepatocytes SOCE appears to be a more effective way of refilling the stores than $I_{\text{CAN}}$ (Gregory et al. 2003).

The FFA-sensitive $I_{\text{CAN}}$ is enhanced by DA and contributes to sustained pacemaker oscillations. We showed that $I_{\text{CAN}}$ plays an important role in DA-induced AB bursting. First, FFA terminated ongoing dopamine-evoked oscillations and blocked $I_{\text{CAN}}$ with an IC$_{50}$ of 24 μM (Fig. 5A). FFA is routinely used at 100–500 μM to reduce $I_{\text{CAN}}$ in both invertebrates and vertebrates (Derjean et al. 2005; Ghamari-Langroudi and Bourque 2002; Green and Cottrell 1997; Lee and Tepper 2007; Morisset and Nagy 1999; Pace et al. 2007a; Partridge and Valenzuela 2000). At these high concentrations, above 100 μM, FFA has multiple side effects, including partial inhibition of calcium channels and NMDA.
receptors (Wang et al. 2006) and induction of calcium release from ER (Gardam et al. 2008; Lee et al. 1996; Shaw et al. 1995) and mitochondria (Tu et al. 2009). We avoided these side effects by using much lower concentrations (below 10 μM). In our system, FFA block causes the membrane potential to stabilize around -40 to -42 mV (Fig. 5A), near the upper voltage limit of the slow oscillatory wave. This end-point depolarization was also seen in the experiments in which we blocked AB oscillations by disruption of intracellular calcium dynamics (Fig. 1). While we do not understand the basis for this depolarization, it may be the result of calcium compartmentalization into separate domains and limiting the calcium supply to calcium-activated potassium [K(Ca)] channels that normally stabilize the potential near its limiting value.

The cellular calcium dynamics (Fig. 1) shows that AB oscillations were blocked by disruption of intracellular calcium compartmentalization into separate domains and limited the calcium supply to calcium-activated potassium [K(Ca)] channels that normally stabilize the potential near its limiting value. This end-point depolarization was also seen in the experiments in which we blocked AB oscillations by disruption of intracellular calcium dynamics (Fig. 1). While we do not understand the basis for this depolarization, it may be the result of calcium compartmentalization into separate domains and limiting the calcium supply to calcium-activated potassium [K(Ca)] channels that normally stabilize the potential near its limiting value. This was not the main cause of the cessation of bursting, as the AB neuron also failed to burst upon artificial hyperpolarization to its initial resting potential.

A second argument for the role of I_{CAN} in DA-evoked AB bursting is that DA significantly enhances this current’s maximal conductance and slows its deactivation rate. The I_{CAN} peak amplitude almost doubled during DA application (Fig. 5, A and B), despite DA inhibition of I_{Ca(V)j} (Johnson et al. 2003), which would normally act to reduce I_{CAN}. Monoamine-induced modulation of I_{CAN} was previously described in the Aplysia R15 neuron (Lotshaw et al. 1986) and Helix bursting neurons (Partridge et al. 1990), as well as in the DG neuron of the Cancer STNS (Zhang et al. 1995). In the AB neuron, DA could modulate I_{CAN} either directly, via second messenger-mediated modulation of these channels, or indirectly by elevating intracellular calcium levels, which would interact cooperatively with calcium entering from the extracellular space; our data suggest that at least this second mechanism occurs. Alternatively, DA could slow the rate of reuptake of calcium into intracellular stores after entering via voltage-activated channels, thus increasing the maximal I_{CAN} and slowing its deactivation rate.

Model of DA-induced bursting of the lobster pyloric pacemaker neuron. Based on previous work and our data, we suggest the following model of DA-induced bursting in AB neurons (Fig. 6).

1) DA binds to D_{1A} or D_{1B} receptors and increases the intracellular [Ca^{2+}] in the fine neuropil. The ER calcium channels and the SERCA play a major part in this process. IP_{3}Rs are likely activated via the G_{q}-PLC-phosphatidylinositol 4,5-bisphosphate (PIP_{2}) pathway with a subsequent activation of additional IP_{3}Rs and RyRs as calcium as calcium is released from the ER.

2) This rise in intracellular calcium activates I_{CAB}. DA increases the peak current amplitude and slows the rate of deactivation of the current, probably due to elevated levels of cytoplasmic calcium, although a direct modulatory action on the channels is also possible.

3) I_{CAB} activation acts as a ramp current in addition to I_{h} to depolarize the cell after the termination of the previous burst. This ramp brings the cell to voltage threshold to activate other inward currents possibly including the DA-insensitive portion of I_{Ca(V)}, to initiate additional calcium entry and the full burst. This additional influx of Ca^{2+} could also recruit more I_{CAB} during the burst phase to contribute to the burst itself.

4) Depolarization and high intracellular calcium activate calcium-dependent outward currents, such as I_{K(Ca)}, which must have either slower activation kinetics or a higher calcium concentration dependence compared with I_{CAB}. Other currents, such as sodium-sensitive potassium currents [I_{K(Na)}] or pump currents, may also contribute to this accumulation of outward currents. The outward currents eventually prevail over the inward currents driving the depolarization; this repolarizes the cell, ending the cycle.

5) Extracellular calcium is essential for replenishment of the intracellular calcium stores and is carried inside via I_{CAB}, I_{Ca(V)}, and/or SOCE.

6) In addition to these effects, previous work in our laboratory has shown that DA also reduces the tonic leak current, which will make the neuron electrically tighter and more compact, enhancing the effects of the rather small I_{CAB} and other subthreshold currents (Fig. 6B). DA reduces the transient potassium current (Peck et al. 2001), which would normally counteract the ramp I_{CAB} current. DA also enhances the hyperpolarization-activated inward current I_{h} (Peck et al. 2006), which would also contribute to the depolarizing ramp driving regenerative oscillations in AB. Paradoxically, DA also enhances a slowly activating and deactivating inward-rectifying I_{K(Na)} (Gruhn et al. 2005), which may act to restrict the maximal spike frequency of the oscillating AB neuron (Harris-Warrick and Johnson 2010; Fig. 6).

Multiple pacemaking mechanisms. There are multiple possible ionic mechanisms to generate bursting in neurons, and the relative contribution of each may vary under different conditions (Harris-Warrick and Flamm 1987; Harris-Warrick and Johnson 2010; Peña et al. 2004). In the vertebrate respiratory central pattern generator (CPG), there may exist different groups of pacemaker neurons, each with a distinct rhythm-
genic mechanism—either sodium dependent or calcium dependent (Del Negro et al. 2002; Pace et al. 2007a, 2007b; Peña et al. 2004). Our work shows how DA evokes AB bursting by a calcium-dependent mechanism (Harris-Warrick and Flamm 1987). However, the same AB neuron uses a completely different mechanism to induce bursting in the presence of 5-HT, which is driven by a sodium-dependent mechanism (Harris-Warrick and Flamm 1987; Kadiri and Harris-Warrick, unpublished observations). Thus if in the respiratory CPG there may be different groups of pacemaker neurons in the same CPG, in the pyloric CPG a single pacemaker neuron can employ two quite distinct ionic mechanisms to drive rhythmic behaviors.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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DA-INDUCED PACEMAKING


