Role of Membrane Potential in Calcium Signaling During Rhythmic Bursting in Tritonia Swim Interneurons

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Hill ES, Katz PS. Role of membrane potential in calcium signaling during rhythmic bursting in Tritonia swim interneurons. J Neurophysiol 97: 2204–2214, 2007. First published January 17, 2007; doi:10.1152/jn.01244.2006. Rhythmic bursting in neurons is accompanied by dynamic changes in intracellular Ca2+ concentration. These Ca2+ signals may be caused by membrane potential changes during bursting and/or synaptic inputs. We determined that membrane potential is responsible for most, if not all, of the cytoplasmic Ca2+ signal recorded during rhythmic bursting in two neurons of the escape swim central pattern generator (CPG) of the mollusk, Tritonia diomedea: ventral swim interneuron B (VSI) and cerebral neuron 2 (C2). Ca2+ signals were imaged with a confocal laser scanning microscope while the membrane potential was recorded at the soma. During the swim motor pattern (SMP), Ca2+ signals in both neurons transiently decay in secondary than in primary neurites. VSI and C2 were then voltage-clamped at the soma, and each neuron’s own membrane potential waveform recorded during the SMP was played back as the voltage command. In all regions of VSI, this completely reproduced the amplitude and time course of Ca2+ signals observed during the SMP, but in C2, the amplitude was lower in the playback experiments than during the SMP, possibly due to space clamp problems. Therefore in VSI, the cytoplasmic Ca2+ signal during the SMP can be accounted for by its membrane potential excursions, whereas in C2 the membrane potential excursions can account for most of the SMP Ca2+ signal.

INTRODUCTION

During the production of rhythmic motor patterns by central pattern generators (CPGs), intracellular Ca2+ concentration in CPG neurons can play a role in the generation of bursting (Falcke 2000; Roussel et al. 2006) and in homeostatic regulation of cellular properties that allow bursting to occur (Marder and Goaillard 2006). The level of intracellular Ca2+ can be affected by the opening of voltage-gated Ca2+ channels and by synthetically driven inputs that lead to increases in intracellular Ca2+. Synaptically driven inputs (including neuromodulatory inputs) can cause a direct influx of Ca2+ through ligand-gated channels (Fucile 2004; Nayak et al. 1999), alter Ca2+ influx through voltage-gated channels (Kloppenburg et al. 2000; Ladewig et al. 2004; Oikawa et al. 2005) or alter the release of Ca2+ from intracellular stores (Berridge 1998). Determining the contributions of these mechanisms to Ca2+ signaling in CPG neurons during bursting is a first step toward understanding the control of Ca2+ dynamics during motor pattern generation.

The CPG underlying the escape swimming behavior of the opisthobranch mollusk Tritonia diomedea provides an opportunity to study Ca2+ signaling in a well-defined neuronal network. The CPG circuit contains three types of interneurons: the serotonergic dorsal swim interneurons (DSIs), cerebral interneuron 2 (C2), and ventral swim interneuron B (VSI) (Fig. 1). The swim is an episodic behavior; the neurons do not produce the rhythmic swim motor pattern (SMP) until it is triggered by sensory input. During the SMP, the CPG neurons fire three to eight rhythmic bursts of action potentials with a period of ~7 s. The Tritonia swim CPG has been described as a network oscillator; none of the neurons can generate rhythmic membrane potential oscillations in isolation, rather rhythmic activity arises through network interactions (Getting 1989).

The swim CPG contains intrinsic neuromodulation: serotonin, released from the DSIs, modulates the synaptic output strength of both C2 and VSI (Katz and Frost 1995a,b; Katz et al. 1994; Sakurai and Katz 2003). In fact, serotonin is necessary and sufficient for initiation of the SMP: application of a serotonin receptor antagonist prevents the SMP from occurring, whereas intracellular stimulation of individual DSIs or bath application of serotonin can initiate an SMP (Fickbohm and Katz 2000; McClellan et al. 1994). It is possible that the release of serotonin from the DSIs or the release of other unidentified neuromodulators could allow the SMP to occur by contributing to Ca2+ signaling in VSI and C2.

In this study, we first observed the Ca2+ signals in VSI and C2 during the SMP. Then, to determine the proportion of those Ca2+ signals that could be accounted for by membrane potential excursions during the SMP, we voltage-clamped VSI and C2 in turn and measured the Ca2+ signals produced by playing back the voltage recordings of these neurons during the SMP as the voltage command. Thus VSI and C2 reproduced the same membrane potential excursions, but did not receive the same synaptic and neuromodulatory inputs as during the SMP. Our data show that SMP membrane potential excursions can completely account for the SMP Ca2+ signals in VSI but not in C2.

Portions of this work have been published previously in abstract form (Hill and Katz 2005).

METHODS

Experiments were performed on Tritonia diomedea obtained from Living Elements (Delta, British Columbia, Canada). Animals were maintained in artificial recirculating, chilled (10°C) seawater prior to experiments. Dissection protocols were as described earlier (Getting 1989).

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et al. 1980; Katz and Frost 1995a). The isolated brain, consisting of the fused cerebropleural ganglion and the pedal ganglia, was pinned to a silicone elastomer (Sylgard)-coated 35-mm petri dish, desheathed, and superfused with chilled (10°C) normal saline, containing (in mM) 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10 d-glucose, and 10 HEPES, pH 7.4.

Neurons were impaled with glass microelectrodes filled with 3 M KCl (8–12 MΩ). VSI was identified on the basis of the following characteristics: soma location (1 cell layer below the surface of the ventral side of the pleural ganglion), activity during the SMP, intrinsic characteristics: soma location (1 cell layer below the surface of the ventral side of the pleural ganglion), and comprises the interneurons DSI, C2, and VSI. The isolated brain, consisting of the fused cerebropleural ganglion and the pedal ganglia, was pinned to a silicone elastomer (Sylgard)-coated 35-mm petri dish, desheathed, and superfused with chilled (10°C) normal saline, containing (in mM) 420 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 10 d-glucose, and 10 HEPES, pH 7.4.

Neurons were impaled with glass microelectrodes filled with 3 M KCl (8–12 MΩ). VSI was identified on the basis of the following characteristics: soma location (1 cell layer below the surface of the ventral side of the pleural ganglion), activity during the SMP, intrinsic characteristics: soma location (1 cell layer below the surface of the ventral side of the pleural ganglion), and comprises the interneurons DSI, C2, and VSI.

RESULTS
Rhythmic Ca²⁺ signals during the SMP showed spatial heterogeneity in VSI

The VSI soma is located in the pleural ganglion, and it has an extended geometry with at least two distinct arborizations (Fig. 1B). A primary neurite extends anteriorly and projects laterally to the ipsilateral pedal ganglion. VSI has secondary neurites proximal to the soma in the pleural ganglion and secondary neurites in the pedal ganglion. It is not known whether VSI receives inputs in both regions and it is not known where VSI synapses with its postsynaptic follower neurons.
During the SMP, VSI and the other swim CPG neurons fire bursts of action potentials riding on underlying membrane potential oscillations (Fig. 2, bottom). Ca$^{2+}$ signals imaged during the SMP showed rhythmic oscillations that corresponded to the action potential bursts recorded at the soma of VSI (Fig. 2). Rhythmic Ca$^{2+}$ signals were recorded in both proximal (pleural ganglion; Fig. 2A) and distal (pedal ganglion) regions (Fig. 2B) of VSI.

There were consistent differences between the Ca$^{2+}$ signals imaged in primary and secondary neurites in both the proximal and distal regions of VSI. The Ca$^{2+}$ signals in primary neurites decayed more slowly than those of secondary neurites in both proximal and distal regions of VSI, and the Ca$^{2+}$ signals in distal neurites decayed more rapidly than in the corresponding proximal neurites [i.e., distal primary neurites vs. proximal primary neurites, and distal secondary neurites vs. proximal secondary neurites; Table 1, $P < 0.05$ for all the preceding pair-wise comparisons (Kruskal-Wallis 1-way ANOVA on ranks with Student-Newman-Keuls multiple pair-wise comparisons)]. The peak amplitude of the Ca$^{2+}$ signal was greater in VSI secondary neurites than in primary neurites in the proximal region. Furthermore, the Ca$^{2+}$ signal in primary neurites was significantly greater in distal than in proximal regions [Table 1, $P < 0.05$ for both pair-wise comparisons (Kruskal-Wallis 1-way ANOVA on ranks with Student-Newman-Keuls multiple pair-wise comparisons)].

**Membrane potential during the SMP entirely accounted for the Ca$^{2+}$ signals in VSI**

To determine the extent to which the Ca$^{2+}$ signal in VSI was caused by the membrane potential excursions during the SMP, VSI was voltage-clamped (dSEVC) and its own membrane potential recorded during the SMP was played back as the voltage command. In this way, the VSI membrane potential reproduced the same pattern that it exhibited during the SMP without the influence of synaptic and neuromodulatory inputs. This protocol effectively reactivated VSI without activating the rest of the network to produce the SMP (Fig. 3). In this example, two rhythmically active pedal ganglion neurons were recorded during the SMP: a ventral flexion neuron (VFN) and a dorsal flexion neuron A (DFN-A) (Hume et al. 1982). When the VSI swim membrane potential excursions were recreated via voltage-clamp (Fig. 3), the DFN-A no longer displayed rhythmic activity. The VFN was directly driven by VSI but showed greatly reduced activity compared with its activity during the SMP. Extracellularly recorded rhythmic activity was also greatly curtailed, leaving only the VSI action potentials in pedal nerve 6. Similar results were observed in three

![FIG. 2. VSI Ca$^{2+}$ signals in proximal (A) and distal (B) regions during a swim motor pattern (SMP). A and B: SMP was elicited by a nerve shock (↑). Differences in temporal dynamics of Ca$^{2+}$ signals in primary vs. secondary neurites were observed in both proximal (A) and distal (B) regions of VSI. In the primary neurite, the Ca$^{2+}$ signals summated and stayed above the basal level long after the nerve shock. In the secondary neurites, the Ca$^{2+}$ signals did not summate and returned back to basal levels faster than in the primary neurite.](image-url)

### TABLE 1. Decay time constant and peak amplitude of Ca$^{2+}$ signals in VSI and C2

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<th>VSI</th>
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<td>Decay time constant, s</td>
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<td>Peak amplitude, %ΔF/F</td>
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<tr>
<td>Decay time constant, s</td>
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<td>28.0 ± 5.7</td>
<td>9.4 ± 1.8</td>
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<tr>
<td>Peak amplitude, %ΔF/F</td>
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<td>NA</td>
<td>157.8 ± 28.1</td>
<td>161.2 ± 21.3</td>
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All values represent means ± SE.
preparations. These results suggest that replaying the swim motor pattern in VSI did not activate the CPG.

Replaying the VSI membrane potential excursions from the SMP fully reproduced the Ca\textsuperscript{2+} signals recorded during the SMP in both the proximal (Fig. 4A) and distal regions of VSI (Fig. 4B). Even the difference in the time course of the Ca\textsuperscript{2+} signals in primary and secondary neurites was reproduced by the voltage playback. To quantify the signal, the area under the $F/F_0$ curve was integrated from the time of the stimulus to 60 s after the end of the SMP. There were no significant differences between the Ca\textsuperscript{2+} signals produced by playback of the membrane potential and those imaged during the SMP [Fig. 4, A and B; $P > 0.05$ in all cases (paired $t$-test)].

Contribution of spikes and underlying membrane potential oscillations to the Ca\textsuperscript{2+} signals in VSI

To determine the relative contribution to the Ca\textsuperscript{2+} signal of the action potentials and the underlying membrane potential depolarization during the SMP, we used each separately as the

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**FIG. 3.** Replaying the VSI SMP voltage waveform under voltage clamp (VC) did not elicit an SMP. A: during the SMP, VSI, and 2 pedal ganglion neurons (a VFN and a DFN-A) are rhythmically active. Rhythmic activity was also observed in pedal nerve 6. B: voltage clamping VSI and using its own SMP waveform as the voltage command caused VSI to exhibit voltage signals identical to those during the SMP; however, the DFN-A was not rhythmically active, and the VFN (directly driven by VSI) showed greatly reduced activity compared with its activity during the SMP, and no rhythmic activity was observed in pedal nerve 6 (VSI’s spikes can be observed in pedal nerve 6). Thus replaying VSI’s SMP voltage waveform in VC did not elicit an SMP.

**FIG. 4.** Using the SMP voltage signal as the voltage command fully reproduced the SMP Ca\textsuperscript{2+} signals in primary and secondary neurites in the proximal (A) and distal (B) regions of VSI. Ai: SMP (black traces) and VC (blue traces) Ca\textsuperscript{2+} signals from proximal regions superimposed. Aii: there was no significant difference in the integral [$\Delta F/F_0$*s] (s = seconds) values between the SMP and VC Ca\textsuperscript{2+} signals in both the primary and secondary neurites in the proximal regions of VSI [$P > 0.05$ for both (paired $t$-test)]. Bi: SMP (black traces) and VC (blue traces) Ca\textsuperscript{2+} signals in distal regions superimposed. Bii: there was no significant difference in the integral [$\Delta F/F_0$*s] values between the SMP and VC Ca\textsuperscript{2+} signals in both the primary and secondary neurites in the distal regions of VSI [$P > 0.05$ for both (paired $t$-test)].
voltage command and measured the respective Ca\textsuperscript{2+} signals (Fig. 5). In the different regions of VSI, there were differences in the extent to which the spikes alone contributed to the Ca\textsuperscript{2+} signals. In proximal primary neurites, the action potential waveforms alone reproduced 75.5% of the integrated amplitude of Ca\textsuperscript{2+} signals produced by the full SMP waveform (n = 2), whereas in the proximal secondary neurites, spikes alone accounted for 60.1% of the signal (n = 2; Fig. 5A, i, green traces, and ii). In proximal regions of VSI, the underlying SMP membrane potential oscillations, produced Ca\textsuperscript{2+} signals that were 35.0 and 31.7% of the Ca\textsuperscript{2+} signals produced by the full SMP waveform (primary and secondary neurites, respectively; both n = 3; Fig. 5A, i, magenta traces, and ii). Thus most of the Ca\textsuperscript{2+} signal during the SMP in the proximal regions of VSI was spike-mediated, but the subthreshold membrane potential oscillations contributed substantially.

In distal regions, the spikes alone accounted for an even greater percentage of the Ca\textsuperscript{2+} signal, whereas the subthreshold membrane potential oscillations played almost no role. When just the action potential waveforms were played back as the voltage command, 74.1% of the signal produced by the full SMP waveform was observed in the primary neurites (n = 3) and 95.5% in the secondary neurites (n = 4; Fig. 5B, i, green traces, and ii). Replaying the underlying SMP membrane potential signal produced just 11.6 and 5.3% of the Ca\textsuperscript{2+} signals produced by the full SMP waveform (primary and secondary neurites, respectively; n = 3 and n = 4, respectively; Fig. 5B, i, magenta traces, and ii). Thus the Ca\textsuperscript{2+} signal in the distal regions of VSI appears to be primarily spike-mediated.

Rhythmic Ca\textsuperscript{2+} signals showed spatial heterogeneity in C2 during the SMP

Unlike VSI, C2 has its soma in the cerebral ganglion and projects contralaterally to the pedal ganglion. It has almost no arborizations in the cerebral ganglion but has extensive arborizations in the contralateral pedal ganglion. As with VSI, it is unknown where C2 receives synaptic inputs or contacts its postsynaptic followers.

Rhythmic Ca\textsuperscript{2+} signals were observed in all areas of C2 during the SMP (Fig. 6). In distal (pedal ganglion) regions of C2, the Ca\textsuperscript{2+} signals decayed more rapidly in the secondary neurites than in the primary neurites [Table 1, P < 0.05 (Kruskal-Wallis 1-way ANOVA on ranks with Dunns multiple pairwise comparisons)].
Although the time constant of decay of the signals in the proximal (cerebral ganglion) primary neurite was about twice that of the distal primary neurite, the difference did not show statistical significance [Table 1, \( P/0.05 \) (Kruskal-Wallis 1-way ANOVA on ranks with Dunns multiple pairwise comparisons)]. Furthermore, although there was a trend for the peak amplitude to be largest in the distal secondary neurite and smallest in the proximal primary neurite, there were no significant differences between the peak \( \text{Ca}^{2+} \) signals in any of regions of C2 [Table 1, \( P/0.109 \) (1-way ANOVA)].

Voltage-clamp at the soma could not completely account for SMP \( \text{Ca}^{2+} \) signals in C2

To test whether the \( \text{Ca}^{2+} \) signals imaged in C2 during the SMP could be accounted for by membrane potential excursions, we performed the same procedure as previously described for VSI of voltage-clamping (dSEVC) C2 at the soma and replaying the membrane potential of the neuron recorded during the SMP. As with VSI, this did not cause the network to generate the SMP (Fig. 7). A DSI and a pedal ganglion neuron that burst as a DFN-A (Hume et al. 1982) that had been rhythmically active during the SMP were only minimally affected during the replay of the SMP membrane potential excursions in C2 (Fig. 7; similar results were observed in 3 preparations). Also during the SMP, large rhythmic units were observed in pedal nerve 6 that were absent during the SMP replay in C2. However, C2 propagating action potentials generated by the replay were observed in the recording of pedal nerve 6 (Fig. 7).

Again, as with VSI, the replayed membrane potential reproduced the different time courses of \( \text{Ca}^{2+} \) signals observed during the SMP in the different regions of C2 (Fig. 8, A and B). However, unlike VSI, the amplitudes of the integrated \( \text{Ca}^{2+} \) signals were significantly smaller in the voltage-clamp replay than the original SMP. This was true for \( \text{Ca}^{2+} \) signals in the proximal primary neurite (both ipsi- and contralateral) of C2 [\( P < 0.05 \) for both (paired \( t \)-test); Fig. 8A] and in the distal primary and secondary neurites [\( P < 0.05 \) for both (paired \( t \)-test); Fig. 8B].

Voltage clamping the distal neurite in C2 recreated the SMP \( \text{Ca}^{2+} \) signals

To test whether the differences between the SMP \( \text{Ca}^{2+} \) signals and the voltage-clamp-induced \( \text{Ca}^{2+} \) signals in all
regions of C2 could be caused by space-clamp problems, we sought to voltage clamp the distal primary neurite of C2. In numerous attempts, we managed to impale the distal neurite of C2 only three times, and of those, only once could we maintain the recording long enough to voltage clamp the distal neurite. We first recorded the SMP voltage and Ca\textsuperscript{2+}/H\textsubscript{11001} signals in the distal neurites of C2, and then voltage-clamped the distal primary neurite and recreated the SMP voltage excursions. We found that this completely recreated the SMP Ca\textsuperscript{2+}/H\textsubscript{11001} signals (Fig. 9), something that we never observed while voltage clamping at the soma of C2. Although we were able to perform this technically difficult experiment only once, this result was clear and suggests that space-clamp issues rather than synaptic inputs could be the cause of the discrepancies between the SMP Ca\textsuperscript{2+}/H\textsubscript{11001} signals observed while clamping C2 at the soma.

Contribution of spikes and underlying membrane potential oscillations to the Ca\textsuperscript{2+} signals in C2

The proportion of the Ca\textsuperscript{2+} signal that can be accounted for by the action potentials in C2 was less than in VSI. In proximal regions of C2, the spikes alone produced only 12.6% and 17.9% (ipsi- and contralateral, respectively, both \(n = 5\)) of the Ca\textsuperscript{2+} signals produced by the full SMP waveform (Fig. 10A, i green traces, ii). The subthreshold membrane potential oscillations accounted for a greater proportion of the Ca\textsuperscript{2+} signal: 42.1% and 36.8% (ipsi- and contralateral, respectively, both \(n = 5\)) in the proximal regions of C2 (Fig. 10A, i, magenta traces, and ii).

In distal regions of C2, the action potentials produced Ca\textsuperscript{2+} signals that were 22.3% (\(n = 4\)) and 37.1% (\(n = 6\); primary and secondary neurites, respectively) of the Ca\textsuperscript{2+} signals produced by the full SMP waveform (Fig. 10A, i green traces, and ii). In distal regions of C2, the subthreshold membrane potential oscillations produced Ca\textsuperscript{2+} signals that were 2.4% (\(n = 4\)) and 17.7% (\(n = 7\); primary and secondary neurites, respectively) of the Ca\textsuperscript{2+} signals produced by the full SMP waveform (Fig. 10B, i, magenta traces, and ii).

DISCUSSION
Ca\textsuperscript{2+} signals rhythmically oscillate during the SMP in both VSI and C2

We recorded rhythmic Ca\textsuperscript{2+} signals in all regions of two Tritonia CPG neurons (VSI and C2) during the SMP. The
The VSI result was somewhat unexpected; we hypothesized that neuropeptidomodulatory or synaptically driven Ca\textsuperscript{2+} signals would contribute to the Ca\textsuperscript{2+} signal recorded during the SMP. Dynamic biochemical signaling has been speculated to play a potentially integral role in the generation of the *Tritonia* SMP (Clemens and Katz 2003). Therefore we expected that VSI would receive at least some synaptic inputs during an SMP that would have produced some of the SMP Ca\textsuperscript{2+} signal. However, our data show that rather than Ca\textsuperscript{2+} driving the voltage oscillations, the voltage oscillations drive the entire Ca\textsuperscript{2+} signal recorded in VSI during rhythmic bursting. Thus in VSI, Ca\textsuperscript{2+} entry via voltage-gated Ca\textsuperscript{2+} channels appears to be responsible for the entire Ca\textsuperscript{2+} signal during rhythmic bursting. It remains unknown whether Ca\textsuperscript{2+} entry via voltage-gated Ca\textsuperscript{2+} channels leads to Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from internal stores. Finally, it is possible that synaptic and/or neuropeptidomodulatory inputs during the SMP could contribute to Ca\textsuperscript{2+} signaling in highly restricted microdomains near synaptic terminals.

**Synaptic inputs to C2 during the SMP or space-clamp issues may underlie the discrepancy between the SMP Ca\textsuperscript{2+} signals and those produced by the SMP replay**

In one experiment, we were successful at holding the recording long enough to voltage clamp the distal neurite of C2 and replay the SMP voltage signal. This did recreate the SMP Ca\textsuperscript{2+} signals in both the primary and secondary neurites of C2, something we never observed with voltage clamp at the soma. This result suggests that space-clamp issues could be the cause of the discrepancy between the SMP Ca\textsuperscript{2+} signals and those produced by replaying the SMP voltage signal at the soma of C2. However, because we were not able to voltage clamp the distal neurite of C2 more than once, these results must be interpreted with caution.

It remains a possibility that synaptic inputs to C2 during the SMP could contribute to Ca\textsuperscript{2+} signaling. First of all, a neurotransmitter/modulator (such as serotonin) could activate metabotropic receptors, leading to second-messenger cascades that result in localized Ca\textsuperscript{2+} signals (i.e., release of Ca\textsuperscript{2+} from internal stores or Ca\textsuperscript{2+} influx) without causing any voltage signals. In the leech Retzius neuron, activation of metabotropic serotonin receptors induces an influx of Ca\textsuperscript{2+} through Ca\textsuperscript{2+} channels located mainly in the dendrites (Beck et al. 2002). Further, activation of metabotropic receptors can lead to an augmentation of Ca\textsuperscript{2+} influx from internal stores. In lamprey reticulospinal axons, activation of metabotropic glutamate receptors leads to a rapid release of Ca\textsuperscript{2+} from intracellular stores in a Ca\textsuperscript{2+}-dependent manner (Cochilla and Alford 1998). These Ca\textsuperscript{2+} signals would not be reproduced by the voltage signal alone in the absence of the synaptic inputs that triggered the second-messenger cascade that produced or augmented the Ca\textsuperscript{2+} signals. Second, synaptic inputs during the SMP could enhance Ca\textsuperscript{2+} entry into C2 through voltage-gated Ca\textsuperscript{2+} channels. Biogenic amines have been shown to both increase and decrease Ca\textsuperscript{2+} entry through voltage-gated channels. In mouse hypoglossal neurons, serotonin decreases Ca\textsuperscript{2+} influx through voltage-activated Ca\textsuperscript{2+} channels (Ladewig et al. 2004), and in the lobster STG ( stomatogastric ganglion), dopamine increases voltage-activated Ca\textsuperscript{2+} currents in some CPG neurons and decreases it in others (Johnson et al. 2003). Imaging experiments showed that in one lobster STG neuron
Spikes versus underlying waveform—contribution to the full Ca$^{2+}$ signal in proximal (A) vs. distal (B) regions of C2. Ai: using the underlying potential (magenta traces) as the voltage command produced Ca$^{2+}$ signals in the proximal regions of C2 that were stronger than those produced by using the action potentials (green traces) as the voltage command. Aii: in proximal regions of C2, the action potentials alone produced Ca$^{2+}$ signals that were 12.6 and 17.9% (ipsi- and contralateral regions, respectively; both n = 5) of the Ca$^{2+}$ signals produced by the full SMP waveform. In the proximal regions of C2, the underlying potential alone produced Ca$^{2+}$ signals that were 42.1 and 36.8% (ipsi- and contralateral regions, respectively; both n = 5) of the Ca$^{2+}$ signals produced by the full SMP waveform. Bi: in distal regions of C2, using either the action potentials (green traces) or the underlying potential (magenta traces) as the voltage command produced small Ca$^{2+}$ signals. Bii: in the distal regions of C2, the action potentials alone produced Ca$^{2+}$ signals that were 22.3 and 37.1% (primary and secondary neurites, respectively; n = 4) of the Ca$^{2+}$ signals produced by the full SMP waveform. In the distal regions of C2, the underlying potential alone produced Ca$^{2+}$ signals that were 2.4 and 17.7% (primary and secondary neurites, respectively; n = 4 and n = 7, respectively) of the Ca$^{2+}$ signals produced by the full SMP waveform.

Dopamine decreases voltage-activated Ca$^{2+}$ entry in presynaptic terminals (Kloppenburg et al. 2000). Nicotine can also increase Ca$^{2+}$ entry through voltage-gated Ca$^{2+}$ channels (Oikawa et al. 2005). Thus during an SMP, synaptic inputs to C2 could potentially enhance Ca$^{2+}$ entry through voltage-activated Ca$^{2+}$ channels. In fact, recent experiments have shown that stimulation of one DSI (causing synaptic release of serotonin) enhances spike-mediated Ca$^{2+}$ signaling in distal regions of C2 (Hill and Katz 2006). Finally, receptors, such as nicotinic ACh, NMDA, and 5-HT$_3$ receptors, themselves have been shown to be permeable to Ca$^{2+}$ (Cochilla and Alford 1999; Fucile 2004; Nayak et al. 1999; Single and Borst 2001). Synaptic inputs to C2 during an SMP could thus directly lead to Ca$^{2+}$ influx into C2 via Ca$^{2+}$-permeable receptors.

VSI and C2 differed in the contribution of action potentials to the Ca$^{2+}$ signals. In the distal regions of VSI, the action potentials alone accounted for ~75–95% of the Ca$^{2+}$ signal, whereas the underlying potential had almost no effect. In proximal regions of VSI, the underlying potential contributed more to the Ca$^{2+}$ signal, but still less than the contribution of the action potentials. These differences could reflect regional differences in the distribution of Ca$^{2+}$ channels: for example, in distal regions there could be a greater density of high-threshold Ca$^{2+}$ channels, whereas in proximal regions of VSI, the relative density of low-threshold Ca$^{2+}$ channels could be higher.

In proximal regions of C2, the situation was reversed—the underlying potential contributed more to the Ca$^{2+}$ signal than did the action potentials. These data indicate that in proximal regions of C2 there may be a greater density of low-threshold Ca$^{2+}$ channels than high-threshold Ca$^{2+}$ channels. Furthermore, the action potentials recorded in the soma of C2 tended to be smaller than those recorded in the soma of VSI, suggesting that the action potentials may be generated farther away from the soma in C2 than in VSI. In the distal regions of C2,
Ca$^{2+}$ signaling in both VSI and C2 primary and secondary neurites could have functional significance. For instance, presumptive synaptic regions of VSI and C2 (secondary neurites) could have more extensive Ca$^{2+}$ buffering or extrusion than the axon (primary neurite). Because Ca$^{2+}$ is known to play an intimate role in neurotransmitter release, intracellular Ca$^{2+}$ homeostasis may be essential in synaptic regions of VSI and C2. Furthermore, intracellular Ca$^{2+}$ is known to activate Ca$^{2+}$-activated K$^+$ channels, leading to decreased excitability (El Manira and Wallen 2000; Ladewig and Keller 2000). In synaptic regions, it may be important for such a decrease in excitability to end before the next burst of excitatory synaptic inputs arrives. Thus, in terms of recovery kinetics, the secondary neurites of VSI and C2 may represent separate Ca$^{2+}$ compartments from the primary neurite.

Area-specific differences in the amplitude of Ca$^{2+}$ signals in VSI

In VSI, Ca$^{2+}$ signals were larger in the secondary neurites than in the primary neurites in the proximal regions of the neuron, and Ca$^{2+}$ signals were also larger in the distal primary neurite than in the proximal primary neurite. Two studies in other systems, using ratiometric calcium indicators found similar amplitude differences. In lamprey spinal cord neurons, Ca$^{2+}$ signals were found to be larger in distal than proximal dendrites (Viana Di Prisco and Alford 2004), and dendritic Ca$^{2+}$ signals were larger than somatic Ca$^{2+}$ signals in mouse hypoglossal neurons (Ladewig and Keller 2000). In VSI, the amplitude differences could reflect differences in the density of voltage-gated Ca$^{2+}$ channels. Thus, the same voltage signal would elicit a larger Ca$^{2+}$ signal in the areas containing a higher density of voltage-gated Ca$^{2+}$ channels than in areas with a low density of such channels. In C2, there were no significant differences in the amplitude of Ca$^{2+}$ signals in any region of the neuron, possibly indicating a homogeneous distribution of voltage-gated Ca$^{2+}$ channels.

Ca$^{2+}$ signaling in VSI and C2 during the SMP

In conclusion, we have shown that Ca$^{2+}$ signals in two Tritonia CPG neurons during rhythmic bursting are similar in terms of Ca$^{2+}$ dynamics and but may differ in the source of the Ca$^{2+}$ signals. In VSI, the SMP Ca$^{2+}$ signals appear to be purely a result of membrane potential excursions leading to Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ channels. In contrast, synaptic inputs may significantly contribute to Ca$^{2+}$ signaling in C2 during the SMP.

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