Quantitative Investigation of Calcium Signals for Locomotor Pattern Generation in the Lamprey Spinal Cord

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Viana Di Prisco, Gonzalo and Simon Alford. Quantitative investigation of calcium signals for locomotor pattern generation in the lamprey spinal cord. J Neurophysiol 92: 1796–1806, 2004. First published May 12, 2004; 10.1152/jn.00138.2004. Locomotor pattern generation requires the network coordination of spinal ventral horn neurons acting in concert with the oscillatory properties of individual neurons. In the spinal cord, N-methyl-d-aspartate (NMDA) activates neuronal oscillators that are believed to rely on Ca\(^{2+}\) entry to the cytosol through voltage-operated \(\text{Ca}^{2+}\) channels and synaptically activated NMDA receptors. \(\text{Ca}^{2+}\) signaling in lamprey ventral horn neurons thus plays a determinant role in the regulation of the intrinsic membrane properties and network synaptic interaction generating spinal locomotor neural pattern activity. We have characterized aspects of this signaling quantitatively for the first time. Resting \(\text{Ca}^{2+}\) concentrations were between 87 and 120 nM. \(\text{Ca}^{2+}\) concentration measured during fictive locomotion increased from soma to distal dendrites [from 208 ± 27 (SE) nM in the soma to 335 ± 41 nM in the proximal dendrites to 457 ± 68 nM in the distal dendrites]. We sought to determine the temporal and spatial properties of \(\text{Ca}^{2+}\) oscillations, imaged with \(\text{Ca}^{2+}\)-sensitive dyes and correlated with fluctuations in membrane potential, during lamprey fictive locomotion. The \(\text{Ca}^{2+}\) signals recorded in the dendrites showed a great deal of spatial heterogeneity. Rapid changes in \(\text{Ca}^{2+}\)-induced fluorescence coincided with action potentials, which initiated significant \(\text{Ca}^{2+}\) transients distributed throughout the neurons. \(\text{Ca}^{2+}\) entry to the cytosol coincided with the depolarizing phase of the locomotor rhythm. During fictive locomotion, larger \(\text{Ca}^{2+}\) oscillations were recorded in dendrites compared with somata in motoneurons and premotor interneurons. \(\text{Ca}^{2+}\) fluctuations were barely detected with dyes of lower affinity providing alternative empirical evidence that \(\text{Ca}^{2+}\) responses are limited to hundreds of nanomolars during fictive locomotion.

INTRODUCTION

Central pattern generators (CPGs) are networks of neurons the activity of which underlies many vital behaviors like digestion, circulation, respiration, and locomotion (Arshavsky et al. 1997; Calabrese 1995; Grillner et al. 2001; Marder 2000; Selverston 1993). The interplay of intrinsic properties of neurons and their synaptic interactions determines the operation of these networks (Arshavsky et al. 1997; Grillner and Wallén 1985, 2002). Both network connectivity and neuronal intrinsic properties depend on \(\text{Ca}^{2+}\) signaling (Bootman et al. 2002; Ghosh and Greenberg 1995; Zucker 1999). \(\text{Ca}^{2+}\) entry not only determines synaptic transmitter release, playing a fundamental role in communication between cells, but also is a critical factor that affects intrinsic membrane properties. A deeper understanding of CPGs requires knowledge of how molecular components determine the properties of individual neurons, and consequently, the operation of the network in which they are embedded (Dale and Kuenzi 1997).

The CPG that coordinates muscle contractions during lamprey swimming can be recorded in the spinal cord in vitro (Grillner et al. 1998) after the application of exogenous glutamate receptor agonists like \(\text{N}\)-methyl-d-aspartate (NMDA). This pattern, “fictive” locomotion, is defined as the presence of bursts of action potentials in the ventral roots that alternate between the left and right sides of the spinal cord at any given segment (Cohen and Wallén 1980). Several mechanisms underlie these oscillating bursts. A network of reciprocally connected excitatory and inhibitory interneurons sequentially depolarizes and hyperpolarizes the motoneuron pool (Buchanan and Grillner 1987; Ekeberg et al. 1991). Burst termination in these interneurons has been attributed to the secondary effects of \(\text{Ca}^{2+}\) entry through voltage-gated \(\text{Ca}^{2+}\) channels and NMDA receptors and to spike adaptation due to summation of spike afterhyperpolarizations by \(\text{Ca}^{2+}\) activation of a potassium current (\(I_{\text{KCa}}\)) (El Manira et al. 1994). Similarly, exogenous NMDA application activates intrinsic neuronal oscillators that are believed to require \(\text{Ca}^{2+}\) entry through the NMDA receptor in lamprey motoneuron and in other vertebrates (Hochman et al. 1994; Reith and Sillar 1998; Tell and Jean 1993; Wallén and Grillner 1987). Thus in tetrodotoxin (TTX), to inhibit intracellular coordination, NMDA receptor-mediated oscillations are recorded in lamprey ventral horn neurons (Sigvardt et al. 1985). Burst termination is also strongly modified by the influence of stretch-sensitive neurons (Viana Di Prisco et al. 1990) while several neuromodulators, serotonin, dopamine, and GABA, are thought to act on calcium channels modifying intracellular \(\text{Ca}^{2+}\) concentration or on the K⁺ currents that \(\text{Ca}^{2+}\) entry may activate (Kemnitz 1997; Matsushima and Grillner 1992; Matsushima et al. 1993).

Qualitative changes in intracellular \(\text{Ca}^{2+}\) have been very briefly described in lamprey motoneurons, dendrites, and axons during fictive swimming (Bacskai et al. 1995) but the sources, types of neurons, subcellular locations, and amplitudes of somatic and dendritic cytosolic-free \(\text{Ca}^{2+}\) fluctuations remain unresolved. Elevation in intracellular \(\text{Ca}^{2+}\) concentration may be due to \(\text{Ca}^{2+}\) influx through voltage-dependent \(\text{Ca}^{2+}\) channels, synaptic glutamate-activated channels, in particular NMDA receptors, and \(\text{Ca}^{2+}\) release from internal stores. Spatial heterogeneity of \(\text{Ca}^{2+}\) signals may have important implications for motor pattern generation. The relative locations of sources of \(\text{Ca}^{2+}\), and the K⁺ channels at which this \(\text{Ca}^{2+}\) acts, will impact the \(\text{Ca}^{2+}\) concentrations necessary for neuronal
oscillation and the time course of the oscillations (Jahromi et al. 1999; Marrion and Tavalin 1998; Sah and Bekkers 1996). A better understanding of Ca\(^{2+}\) dynamics in CPG neurons is clearly needed (Ivanov and Calabrese 2000; Kloppenburg et al. 2000).

Although Ca\(^{2+}\) entry to neurons of vertebrate central pattern generators is considered a vital component of the operation of all neurons involved in this activity, we know very little of this phenomenon. We have therefore sought to determine the temporal and spatial properties of Ca\(^{2+}\) entry during fictive locomotion in the lamprey spinal cord, a neural system the basic components of which are believed to be retained in higher vertebrates (Burke et al. 2001; Cohen 1992; Pribe et al. 1997; Rossignol and Dubuc 1994). We wished to monitor Ca\(^{2+}\) transients evoked during fictive locomotion in the somata and dendrites of motoneurons and neurons of the CPG. CPG neurons are the population of interneurons that similar to motoneurons show oscillating membrane potentials during fictive locomotion. To load the neurons with Ca\(^{2+}\)-sensitive dye, we have used techniques that are both invasive and noninvasive to these neurons. Noninvasive loading ensured that the recording technique did not interfere with the final Ca\(^{2+}\) concentration to validate absolute Ca\(^{2+}\)-transient amplitudes prior to and during fictive locomotion. Invasive recording, in which dye is loaded into the neurons through a recording microelectrode, enabled correlations to be made between electrophysiological activity and Ca\(^{2+}\) transients. Preliminary results have been published in abstract form (Viana Di Prisco et al. 2001).

**METHODS**

Experiments were performed on the isolated spinal cords of both adult and larval lampreys (*Petromyzon marinus* and *Ichthyomyzon unicuspis*). The animals were anesthetized with tricaine methanesulfonate (MS-222; 100 mg/l; Sigma Chemical, St. Louis, MO), decapitated, and dissected in a cold saline solution (Ringer) of the following composition (in mM): 100 NaCl, 2.1 KCl, 2.6 CaCl\(_2\), 1.8 MgCl\(_2\), 4 glucose, 5 HEPES, adjusted to a pH of 7.60 (modified from Wickelgren 1977). The skin and muscle were removed. The spinal cord was isolated and removed from the protective *meninx primitiva* and placed in a cooled small-volume chamber with a cover-slip floor that is inserted onto the stage of an upright microscope (Olympus BXW50). The recording chamber was continually superfused with cold oxygenated Ringer (8–10°C). Solutions of pharmacological agents were bath-applied at a perfusion rate of ~1 ml/min. The tissue was held down after attaching it to a platinum staple long enough to record from 15 segments of spinal cord.

**Electrophysiology**

Microelectrode recordings were made conventionally with thin-walled glass sharp micropipettes (20–50 M\(\Omega\)) filled with 1.5 M potassium acetate to which 2.5 mM Ca\(^{2+}\)-sensitive dye (Oregon Green 488 BAPTA-1 dextran or Oregon Green 488 BAPTA-2) was added. Ventral root activity was monitored with suction electrodes.

**Labeling of neurons with Ca\(^{2+}\)-sensitive dyes**

Retrograde labeling of neurons with dextran amine-conjugates of Ca\(^{2+}\)-sensitive dyes has proven to be very effective in the lamprey brain stem preparation (McClellan et al. 1994; Schwartz and Alford 2000). We have adapted this technique to allow dye loading of spinal motoneurons (Takahashi and Alford 2002). Two techniques were used for loading dye into neurons:

**RETROGRADE LABELING OF MOTONEURONS FROM THE MUSCLE WALL.** The animals were anesthetized. A dextran-conjugated Ca\(^{2+}\)-sensitive dye, either 10,000 MW Oregon Green 488 BAPTA-1 dextran; Fluoro-4 dextran; Fura dextran, or 10,000 MW Oregon Green 488 BAPTA dextran (Molecular Probes), was injected into the body musculature (5 mM, 5 \(\mu\)l). The animal was then allowed to recover in a cooled aquarium. After 48 h, the animal was killed under anesthesia, and the spinal cord adjacent to the injection site was removed. Imaging revealed Ca\(^{2+}\)-sensitive dye labeling in motor and sensory neurons of corresponding hemisegments (Fig. 1A).

**INJECTION FROM A SHARP MICROELECTRODE USED TO RECORD VENTRAL HORN NEURONS.** Conventional sharp microelectrode recordings were made from ventral horn neurons under visual observation through a 40x objective lens on an upright microscope. The electrodes were loaded with 2.5 mM Ca\(^{2+}\)-sensitive dye, and by pressure application (200 ms, 2 kPa), rapid dye labeling of the neuronal soma was achieved. The labeled neurons are then left without manipulation for 20 min to allow labeling of the dendrites (Fig. 1B). With this method, we labeled both motoneurons (identified by axon joining a ventral root) and interneurons (identified by axonal projections ipsi or contralateral within the spinal cord). Only interneurons that showed rhythmic activity during fictive locomotion and, hence most likely to be part of the spinal CPG, were included in the study. Because we did not find differences in Ca\(^{2+}\) signals between CPG interneurons and motoneurons their results were pooled.

**Dye calibration**

Spinal motoneurons are readily labeled by retrograde transport of dextran amine-conjugated dye from the trunk musculature (Fig. 1A). Using this technique, it is possible to label, noninvasively, many of the motoneurons in one segment of the spinal cord such that the complete dendritic arborization was visualized. This technique avoids structural damage from microelectrode recording. However, the use of a Ca\(^{2+}\)-sensitive dye may alter Ca\(^{2+}\) responses by buffering free Ca\(^{2+}\) in the cytosol. Estimates of endogenous Ca\(^{2+}\)-buffering capacities vary across neuron types, however, use of 1–10 \(\mu\)M concentrations of fura-2 (\(K_d = 140 \text{ nM}\)) interferes only minimally with endogenous Ca\(^{2+}\) signaling where previously investigated (Neher and Augustine 1992; Tank et al. 1995). We sought to determine that dextran conjugated Oregon Green 488 BAPTA1 (\(K_d = 566 \text{ nM}\) concentrations used in the lamprey were <10 \(\mu\)M to ensure that the dye did not significantly alter Ca\(^{2+}\) dynamics.

Dye concentrations after loading were calibrated by comparing the intensity of neurons loaded with an inert fluorescent dye (Oregon Green 488 dextran) after injection into the trunk musculature (identical concentration and volume to injections with Ca\(^{2+}\)-sensitive dyes, 5 mM, 5 \(\mu\)l) with sample concentrations of the same dye contained within electrodes inserted into the tissue. Microelectrodes containing Oregon Green 488 dextran, at different concentrations from 0.1 to 5 \(\mu\)M dissolved in Ringer solution were sequentially positioned in the tissue and imaged in the same plane as the labeled dendrites and soma with a confocal microscope (\(n = 3\) preparations). This ensured that the image of the calibration pipette and the labeled neurons were at the same optical depth in the tissue (Fig. 1Aii). The fluorescence intensity of the dendrites and soma was compared with that of dye contained in the pipette over the range of concentrations (Fig. 1Aiii). Dye concentrations measured with this technique did not exceed 10 \(\mu\)M. These are concentrations sufficiently low that they should not significantly alter Ca\(^{2+}\) responses in the cells due to problems with buffering (Neher and Augustine 1992). It is also apparent from the confocal image that the motoneurons are labeled to the full extent of their dendrites, and this labeling is resolved in live tissue.
We have demonstrated how readily ventral horn neurons may be labeled with the same dye through the recording microelectrode. In Fig. 1B, a live neuron filled with the Ca\(^{2+}\)-sensitive dye Oregon Green 488 BAPTA1 dextran was imaged on the confocal microscope. The cell was recorded by using a sharp microelectrode containing 1 M K acetate and 1 mM Ca\(^{2+}\) dye. Five pressure pulses (200 ms, 1.5 kPa) were applied to the interior of the pipette to label the neuron, and the image was taken after allowing 15 min for dye diffusion to occur. The injection caused no decrement in input impedance or membrane potential.

**Imaging**

Most imaging was accomplished with a CCD system (Hamamatsu ORCA) mounted onto a compound microscope (Olympus BX50WI) equipped with a rapidly switchable Xenon source (Sutter DG4). For analysis of nonratiometric dyes (e.g., Oregon Green BAPTA-1 or Oregon Green 488 BAPTA1 dextran), fluorescence intensities after background subtraction were normalized to the baseline (prestimulus), giving a baseline value of \(\Delta F/F = 0\). Baseline was defined as the fluorescence at the location analyzed prior to initiation of fictive swimming for retrogradely filled neurons. For neurons filled through the microelectrode, this was defined as the minimum fluorescence during fictive locomotion. For ratiometric imaging, we used dextran-conjugated Fura (Fura dextran) and Ca\(^{2+}\) concentrations calculated using the method of Grynkiewicz et al. (1985). In some cases, dye-filled neurons were imaged on a Biorad MRC 600 confocal microscope (Cochilla and Alford 1998; Schwartz and Alford 2000).

**Calibration of Ca\(^{2+}\)-sensitive dyes**

Ca\(^{2+}\)-fluorescence curves were created from Ca\(^{2+}\) standards obtained form molecular probes. The calibration solutions contained 100 mM KCl and 30 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.2. To achieve a range of Ca\(^{2+}\) concentrations from 0 to 40 \(\mu\)M, 11 buffer solutions were made up comprising 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 mM CaEGTA and corresponding descending concentrations of K\(_{EGTA}\) to maintain constant osmolarity. Fluorescence was measured on the recording microscope after the addition of 5 \(\mu\)M dye (Oregon 488 BAPTA 1 dextran Oregon Green 488 BAPTA2, Fluo-4 dextran, or Fura dextran to the solution). All calibrations were performed at 10°C to conform with the experimental recording temperature.

**RESULTS**

**Ca\(^{2+}\) oscillations recorded during fictive locomotion**

Ventral horn interneurons and motoneurons were labeled by direct injection into the neurons as described in the preceding text (Figs. 1B and 2C; \(n = 9\) cells). Kinetic data that resolved Ca\(^{2+}\) oscillations during fictive locomotion were obtained using the nonratiometric dye Oregon Green 488 BAPTA1 dextran. These data could then be used to relate dynamic changes in Ca\(^{2+}\) concentration to electrophysiological measures of depolarization and spiking.

Fictive locomotion in the lamprey spinal cord comprises alternating ventral root bursting activity between opposite sides of the spinal cord. Motoneurons and premotor interneurons depolarize and may spike in phase with the bursting activity on the same side of the spinal cord. In Fig. 2, a neuron was labeled with Oregon Green 488 BAPTA 1 dextran and the fluorescence imaged at 20 Hz simultaneously to electrophysiological recording of membrane potential and of firing in the adjacent ipsilateral ventral root. Ca\(^{2+}\)
oscillations are observed in both soma and dendrites. In all nine neurons examined, the Ca$^{2+}$ concentration always rose during the depolarizing phase of activity of the recorded neuron, and the simultaneous ipsilateral ventral root burst fell during the hyperpolarizing phase.

Lamprey ventral horn neurons show a great deal of variability in action potential firing frequency during fictive locomotion from zero to three or four action potentials per depolarizing cycle (Buchanan 2001). It is, however, possible to prevent or initiate spiking by injection of DC current through the recording microelectrode. The mean membrane potential of the recorded neuron was controlled by injection of DC current under current-clamp conditions. In Fig. 2A, the neuron was recorded from with no action potentials. Under these conditions, the amplitude of the Ca$^{2+}$ signal varied considerably across different dendritic compartments. The cells were analyzed as three separate compartments as shown in Fig. 2C: the soma, the proximal dendrite (defined as the unbranched dendrite within 50 μm of the soma), and the distal dendrites (comprising branched dendritic compartments distal to the proximal dendrites ≥100 μm from the soma). The amplitude of Ca$^{2+}$ oscillations measured from peak to trough increased from a low at the soma ($\Delta F/F = 0.035 \pm 0.015$) to the proximal dendrites ($\Delta F/F = 0.102 \pm 0.038$) to the distal dendrites ($\Delta F/F = 0.201 \pm 0.066$; where $F$ was defined as the interburst fluorescence after background fluorescence was subtracted, $\Delta F$ as the difference between $F$ and fluorescence at peak). All measurements were the means from nine neurons of the means of at least five locomotor cycles.

The neurons were depolarized with DC current injection to allow spiking in phase with locomotor-induced oscillations (Fig. 2B). The mean amplitude of the Ca$^{2+}$ oscillations was significantly increased during this activity. A single action potential in each cycle increased the Ca$^{2+}$ signal at the soma significantly more than at the distal dendrites ($n = 4$ cells recorded from 4 separate preparations; $P < 0.05$; soma increase of $200 \pm 50\%$, proximal dendrite by $117 \pm 34\%$, distal dendrite $86 \pm 33\%$). This result was consistently seen in all cells examined. This increase in Ca$^{2+}$ signal amplitude was caused by Ca$^{2+}$ entry evoked during the action potential and not by a depolarization-induced alteration in synaptically driven Ca$^{2+}$ entry or a reduction in Ca$^{2+}$ sequestration due merely to the current injection necessary to cause action potentials. This is clear for two reasons: first, the Ca$^{2+}$ signal was only increased in cycles where an action potential occurred; and second, the differential (which represents Ca$^{2+}$ flux into and out of the cytosol) of the Ca$^{2+}$ signal reveals rapid increases in Ca$^{2+}$ concentration that coincide precisely with the timing of action potentials in the neurons (Fig. 3A). Because the fluorescence of the Ca$^{2+}$-sensitive dye is a measure of cytosolic Ca$^{2+}$ concentration, the differential represents Ca$^{2+}$ flux to and from this cellular compartment. The precise timing of action potentials during fictive locomotion and the resulting Ca$^{2+}$ transient was seen throughout the neuron, from soma to distal dendrites, and was seen in all cells examined in which action potentials were recorded.

The differential of the Ca$^{2+}$ signal was also calculated during fictive locomotion when membrane potential oscillations were subthreshold for action potentials. To reduce noise, the graph in Fig. 3B was filtered with a three-point box filter spanning a sampling period of 60 ms. The differential clearly demonstrates the coincidence of cytosolic Ca$^{2+}$ entry with depolarization of the neuron. This result was similar throughout the dendritic tree of all nine neurons examined. We were unable to record significant differences in the timing relationship between depolarizations and Ca$^{2+}$ signals in the neurons examined regardless of whether they were interneurons or motoneurons.

**Locomotor-induced Ca$^{2+}$ transients recorded in retrogradely labeled neurons**

Microelectrode recording from the somata may have interfered with the amplitude of the somatic Ca$^{2+}$ signal, particularly at the

![Fig. 2. Ca$^{2+}$ and membrane potential oscillations during fictive locomotion.](http://jn.physiology.org/)

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impalement site. This would lead to an artifactual apparent distribution of the \( \text{Ca}^{2+} \) transient amplitudes. To determine whether somatic recordings were altered by the recording paradigm, cells were imaged after retrograde labeling of the dye. These recordings confirmed the results using microelectrodes and reveal larger dendritic than somatic \( \text{Ca}^{2+} \) signals essentially the same as the microelectrode recordings (Fig. 4, A and B; and quantified in Fig. 5B). Additionally, it is difficult to normalize the \( \text{Ca}^{2+} \) transient amplitude to fluorescence intensities recorded prior to the application of NMDA and the initiation of fictive locomotion in neurons filled through the microelectrode. This is because slow leakage of dye from the pipette leads to a drift in the resting fluorescence intensity. This drift is not present in neurons labeled retrogradely and so the fluorescence measurements were normalized to resting conditions prior to the initiation of fictive locomotion.

Motoneurons retrogradely labeled with Oregon Green 488 BAPTA1 dextran were imaged before and after initiation of locomotion (by the application of NMDA, 100 or 200 \( \mu \)M, to the superfusate). The fluorescence was normalized to levels recorded in the resting neurons [resting fluorescence = 1; thus fluorescence is expressed as \( \Delta F + F \)/\( F \)]. In the neuron labeled in Fig. 4 the normalized fluorescence recorded along the length of the dendrite outlined in red (Fig. 4Aii) was displayed versus time in a pseudocolor plot. The amplitudes of the peak fluorescence and trough fluorescence was compared along the length of the dendrite and showed very great variance in both these minima and maxima (Fig. 4Ai). In addition, the fluorescence measurements recorded at different locations along the dendritic tree were recorded with time (Fig. 4B). Soma \( \text{Ca}^{2+} \) fluxes were also recorded over a separate time course (Fig. 4C), demonstrating much smaller concentration changes than those recorded in the dendrites. As for data recorded following microelectrode injection with \( \text{Ca}^{2+} \)-sensitive dye there is an increase in \( \text{Ca}^{2+} \) signal amplitude from soma to distal dendrite.

To characterize the spatial distribution of \( \text{Ca}^{2+} \) concentration changes during fictive locomotion, images of fluorescence changes in soma and dendrites were also examined by generating ratio images of data from the troughs and peaks of \( \text{Ca}^{2+} \) measurements during fictive locomotion. Motoneurons were labeled with dextran-conjugated Oregon Green BAPTA 1 and imaged during fictive locomotion initiated by bath application of NMDA (100 or 200 \( \mu \)M) and monitored by ventral root recording (Fig. 5, Ai and Bi). This is the same pool of neurons analyzed in Fig. 4; \( n = 9 \)). False color images presented in Fig. 5, Aii and Bii, were calculated from the ratio of mean fluorescence at the peak and the trough of the oscillations shown in Fig. 5, Ai and Bi, respectively, after subtraction of background fluorescence. The background was masked after identification of fluorescent structures in the images shown in Fig. 5, Aiii and Biii, with a thresholding function. It is clear from this result that substantial variation is seen in the \( \text{Ca}^{2+} \) concentration changes evoked by fictive locomotion recorded across the dendritic tree while substantially lower \( \text{Ca}^{2+} \) concentration changes are seen in the soma and proximal dendrite. This variation in \( \text{Ca}^{2+} \) transient amplitude across the dendritic tree held true for all nine neurons examined and did not correlate to small shifts in focal plane.

**Calibration of \( \text{Ca}^{2+} \) concentrations**

To obtain quantitative data about changes in \( \text{Ca}^{2+} \) concentration during the oscillations, we required a measurement of the \( \text{Ca}^{2+} \) concentration at rest and an estimate of the concentration ranges seen during fictive locomotion. We have used the fura-2 ratiometric method described by Grynkiewicz et al. (1985; see also Neher 1995). Both techniques described in the

**FIG. 3.** \( \text{Ca}^{2+} \) entry to the cytosol occurs synchronously to depolarizing events in the ventral horn neurons. A: action potentials evoke synchronized rapid \( \text{Ca}^{2+} \) entry events. Aii: the same trace recorded in Fig. 2Ai in which the \( \text{Ca}^{2+} \) signal is enhanced during action potentials. Aiii: the differential of the trace in Ai. Data above the solid line indicates \( \text{Ca}^{2+} \) entry to the cytosolic compartment. Below the line indicates removal of \( \text{Ca}^{2+} \) from this compartment. Note that the large abrupt transients coincide precisely with the action potentials in Aiii. Aii: microelectrode recording of the neuronal membrane potential. B: \( \text{Ca}^{2+} \) entry is also synchronized with subthreshold membrane potential oscillations. Bi: the same trace recorded in Fig. 2Bi in which the \( \text{Ca}^{2+} \) signal is recorded during subthreshold locomotor-induced membrane potential oscillations. Bii: the differential of the trace in Bi after 5-point box smoothing function (\( \pm 60 \text{ ms} \)) was applied to the data. \( \text{Ca}^{2+} \) entry is synchronized to membrane potential oscillations. Biii: microelectrode recording of the neuronal membrane potential.
preceding text (see METHODS) were used to load ratiometric dye into spinal neurons. Fura dextran was retrogradely loaded into motoneurons by in vivo injections (5–10 μl, 5 mM) into the muscle wall. Ca²⁺ signals were recorded with excitation wavelengths at 340 and 380 nm in a total of seven neurons in five preparations. A further two neurons were similarly recorded after microinjection with Fura dextran. Resting Ca²⁺ concentrations were calculated from images obtained with 340- and 380-nm excitation on a standard Ca²⁺ calibration buffer kit (Molecular Probes) using the same batch of Fura dye and at the same temperature as the physiological measurements (10°C). Resting Ca²⁺ concentrations (Fig. 6A, unfilled bars) were fairly uniform across the recorded neurons with a mean Ca²⁺ concentration value of 100 ± 15 nM.

Ideally Ca²⁺ concentrations should be calibrated in vitro in the neurons investigated. This is typically achieved by the application of an ionophore to the preparation to permeabilize the preparation to allow maximal and minimal Ca²⁺ binding to be determined. This technique is not feasible in the intact spinal cord because ionophores (e.g., ionomycin) do not penetrate the tissue well. Instead we buffered intracellular Ca²⁺ throughout the spinal cord by the application of the membrane-permeable Ca²⁺ buffer BAPTA-AM. Baseline fluorescence ratios were determined, under these circumstances, for comparison with those obtained with the Ca²⁺ calibration kit. Ratios of emission intensities after excitation at 340 and 380 nm were 0.192 ± 0.03 in neurons in which Ca²⁺ was buffered with BAPTA-AM and 0.196 in the Ca²⁺ calibration kit at 0 Ca²⁺.

Fura dextran recording does not give sufficient temporal resolution in this preparation to investigate dynamic fluctuations in Ca²⁺ concentrations during locomotor activity. At dye...
concentrations sufficiently low to ensure minimal buffering of endogenous Ca$^{2+}$, insufficient fluorescent intensity is resolved to image significant detail with a frame rate more rapid than 5 Hz. However, mean Ca$^{2+}$ concentrations were recorded in various neuronal compartments during resting conditions and then during fictive locomotion evoked by the application of NMDA (100 μM) to the superfusate. The nine retrogradely labeled neurons were analyzed by three compartments for this study, the soma, the proximal dendrite (defined as the unbranched dendrite within 50 μm of the soma), and the distal dendrites (distal to the primary branches ≥100 μm from the soma). Mean Ca$^{2+}$ concentration measured during fictive locomotion increased from soma to distal dendrites (Fig. 6A) similar to the results obtained with the nonratiometric dye. The Ca$^{2+}$ concentration ranged from 208 ± 27 nM in the soma to 335 ± 41 nM in the proximal dendrites to 457 ± 68 nM in the distal dendrites. Mean concentration was measured by calculating the fluorescence ratio obtained by excitation of Fura dextran at 340 and 380 nm over ≥10 frames recorded at 5 Hz during fictive locomotion. Ca$^{2+}$ concentrations were calculated from standard curves obtained as described in METHODS.

**Peak and trough Ca$^{2+}$ concentrations recorded during fictive locomotion**

Estimation of absolute Ca$^{2+}$ concentrations with nonratiometric dyes is not straightforward. However, approximate Ca$^{2+}$ concentrations may be estimated using resting Ca$^{2+}$ concentrations measured with Fura dextran (mean resting concentration ranged from 87 to 120 nM across the three identified neuronal compartments). The affinity of the Ca$^{2+}$ dye (Oregon Green 488 BAPTA1 dextran to Ca$^{2+}$) was obtained from standard buffered Ca$^{2+}$ solutions (Molecular Probes) to which dye was added and Ca$^{2+}$ concentration/fluorescence curves measured at physiological temperature (10°C). The affinity obtained in this way was somewhat lower than published data from the supplier (we calculated the affinity as 566 nM, as compared with 464 nM indicated by Molecular Probes at room temperature. This value varies considerably between batches and with temperature) and a sixfold increase in fluorescence between 0 μM Ca$^{2+}$ bound to a saturating concentration of 40 μM (Fmax/Fmin = 6) was observed. Using the resting Ca$^{2+}$ concentrations calculated from Fura dextran measurements, the k_d of Oregon Green 488 BAPTA1 dextran at 10°C, and the fluorescence measured in control conditions and during fictive locomotion, estimates were made of Ca$^{2+}$ transient concentrations during fictive locomotion (Fig. 5B) using the following equation

$$[\text{Ca}^{2+}] = k_0 \frac{(F - F_{\text{min}})}{(F_{\text{max}} - F)}$$  \hspace{1cm} (1)

Given the ratios of Fmax/Fmin = 6, and Fr/Fmin = 1.9 (calculated from the resting Ca$^{2+}$ concentration obtained with Fura dextran data), and the calibration curve obtained for Oregon Green 488 BAPTA1 dextran, we can calculate [Ca$^{2+}$] (in nM) during locomotion as

$$566 \frac{(1.9(F/F_r)F_{\text{max}} - F_{\text{min}})}{(6F_{\text{max}} - 1.9(F/F_r)F_{\text{min}})}$$  \hspace{1cm} (2)

Where F/F_r = ratio of transient fluorescence during fictive locomotion (Fr) to resting fluorescence (Fr) prior to the addition of NMDA to the superfusate. The Ca$^{2+}$ concentrations estimated using this method during fictive locomotion are similar to those obtained without measuring kinetic information with Fura dextran. All neuronal compartments show Ca$^{2+}$ oscillations during swimming with a simultaneous increase in baseline Ca$^{2+}$. The peak values of the measure (ΔF + F)/F
baseline fluorescence prior to the addition of NMDA, \( \Delta F + F = \text{fluorescence measured during fictive locomotion} \) were at peak 1.19 ± 0.07, 1.34 ± 0.17 and 1.86 ± 0.13 in soma, proximal dendrites and distal dendrites respectively and at minimum in the interburst period 1.15 ± 0.06, 1.16 ± 0.06 and 1.47 ± 0.05 in the same neuronal compartments. These data were calculated from responses during locomotion in 5 neurons from 5 preparations and are shown graphically in Fig. 6B. The left hand vertical axis of that graph shows the equivalent calculated value of Ca\(^{2+}\) concentration given the above equations.

**Imaging Ca\(^{2+}\) with low affinity dyes**

To provide an alternative estimate of the magnitude of Ca\(^{2+}\) oscillations during fictive locomotion, two dyes with significantly lower affinities were used. Low affinity Fluo 4 dextran has an affinity of approximately 3 \(\mu M\) at room temperature. At 10°C its affinity exceeded 5 \(\mu M\). Neurons (n = 3) were filled with this dye both by retrograde labeling and by direct injection through the recording microelectrode. No Ca\(^{2+}\) oscillations or increases in baseline fluorescence were recorded with this dye. A further 5 neurons were labeled with an intermediate affinity dye (Oregon Green 488 BAPTA2). Calibration curves obtained at 10°C indicated an affinity of 1.3 \(\mu M\) and Fmin/Fmax = 18. From this data and the calibration curve the Ca\(^{2+}\) oscillation amplitude during fictive locomotion was predicted to lead to a change in fluorescence of 0.1 \(\Delta F/F\). In two of the four recorded neurons a noticeable Ca\(^{2+}\) oscillation was recorded during locomotion (Fig. 7) The mean oscillation amplitude recorded from peak (amplitude during the burst) divided by the trough (amplitude during the interburst) from all five neurons was 1.1 ± 0.1 (\(\Delta F + F\))/F. This data were not significantly different from the estimate given by higher affinity dyes supporting the results of Ca\(^{2+}\) oscillation amplitude data obtained with those higher affinity dyes and indicating that Ca\(^{2+}\) did not reach a sufficiently high concentration to saturate those dyes.

**DISCUSSION**

**Baseline and transient Ca\(^{2+}\) levels**

Ca\(^{2+}\) oscillations recorded in neurons represent two phases of activity. On the one hand, rising Ca\(^{2+}\) concentrations represent Ca\(^{2+}\) entry into the cytosolic compartment (in which the Ca\(^{2+}\)-sensitive dye is localized) from the extracellular fluid, and from intracellular organelles that function as Ca\(^{2+}\) releasable stores. On the other hand, falling Ca\(^{2+}\) concentrations represent removal of Ca\(^{2+}\) from the cytosolic compartment. Surprisingly little information is available on the kinetics of Ca\(^{2+}\) signaling in neurons that comprise vertebrate central pattern generators (Backsai et al. 1995; Lev-Tov and O’Donovan 1995).

Here we have loaded lamprey spinal motoneurons and interneurons with Ca\(^{2+}\)-sensitive dyes that enable high-speed imaging of physiological Ca\(^{2+}\) signaling during locomotor

Fig. 7. Ca\(^{2+}\) transients measured with a low-affinity Ca\(^{2+}\)-sensitive dye. A: neuron injected with dye (Oregon Green 488 BAPTA2) through the recording microelectrode. The contrast is enhanced over the distal dendritic region to allow visualization. B1: Ca\(^{2+}\) oscillations normalized to the inter-burst minimum in the distal dendrites during fictive locomotion. B2: Ca\(^{2+}\) oscillations recorded in the proximal dendrites. C: Membrane potential of the recorded neuron.
activity. We have measured the magnitude of Ca\(^{2+}\) responses and their baseline concentration. Resting Ca\(^{2+}\) concentrations were found to be between 87 and 120 nM. Mean Ca\(^{2+}\) concentrations recorded during fictive locomotion increased to a high of 450 nM in the distal dendrites. Our absolute estimated value for Ca\(^{2+}\) oscillations during rhythmic activity, are in basic agreement with other reports. For instance Ca\(^{2+}\) concentration increases of 50–200 nM have been reported in mouse hypoglossal motoneurons (Ladewig and Keller 2000). It has been estimated that during locomotor activity recovery of Ca\(^{2+}\) transients must occur on a time scale of tens of milliseconds to avoid a potentially excitotoxic accumulation of basal Ca\(^{2+}\) levels (Palecek et al. 1999). At least in the short term, the endogenous buffering allows motoneurons to rapidly recover from Ca\(^{2+}\) transients at a low energy cost (Palecek et al. 1999). Recent evidence shows that lamprey spinal cord putative CPG neurons and motoneurons are indeed rich in Ca\(^{2+}\) buffering molecules like calbindin and calretinin (Megias et al. 2003).

Spatial heterogeneity of Ca\(^{2+}\) signals due to entry through NMDA channels and other sources

Traditionally much attention has been focused on temporal aspects on membrane potential and spiking activity in the study of rhythmic neural networks, but recently several investigations have aimed at the problem of how synaptic integration from dendritically distributed inputs is conveyed to the soma, and at the functional dynamics of the spatial dendritic branching. For example, modeling studies have shown that bistable oscillatory changes in the dendritic arbor generate different charge transfer to the soma under tonic activation of NMDA conductances (Korogod et al. 2002). Thus here we have investigated the spatial distribution of Ca\(^{2+}\) responses in lamprey ventral horn spinal neurons. We have found that larger Ca\(^{2+}\) concentration changes take place in distal and proximal dendrites, whereas smaller Ca\(^{2+}\) oscillations are present at the soma. This is not at all unexpected because there is evidence that excitatory synaptic contacts and thus glutamate receptors are predominantly located in the dendrites rather than the soma (Moore et al. 1995, 1999).

The spatial distribution of Ca\(^{2+}\) currents likely play an important role in determining synaptic integration and activity control in CPG neurons and motoneurons. Not only presynaptic inputs are distributed along different dendritic compartments but also intrinsic conductances can display a variable spatial distribution over the somato-dendritic membrane. The spatial segregation of Ca\(^{2+}\) entry in dendritic regions and its coupling to Ca\(^{2+}\)-dependent ionic conductances is known to play a key role in determining patterns of neural activity (Hallworth et al. 2003). The intrinsic conductances, in particular Ca\(^{2+}\)-dependent ones, shape the dendritic response to receptor-gated conductances, and provide the dendrites with a dynamic way of regulating integration and spike timing critical for the operation of CPG neurons. Although our amplitude measurements indicated that Ca\(^{2+}\) transients do not exceed 1 μM, we cannot exclude the possibility that mechanisms important for locomotion are activated by very local changes in Ca\(^{2+}\) not resolved by visible light imaging.

Timing of Ca\(^{2+}\) signals and the locomotor rhythm.

In all of the neurons examined Ca\(^{2+}\) entry to the cytosol coincided with the depolarizing phase of the locomotor rhythm. This was true regardless of the location examined within the neuron or whether the depolarization was an action potential or a subthreshold oscillation in membrane potential.

Implications for plasticity in pattern generation

Finally, it is also important to consider that Ca\(^{2+}\) entry oscillations during induced locomotion may be important to other functions beyond the immediate control of the central pattern generation. Various forms of synaptic plasticity such as long-term depression (LTD) and potentiation (LTP) that affect the long-term function of CPGs (Parker and Grillner 1999, 2000; Soto-Trevino et al. 2001; Wolpaw and Tennissen 2001) may rely on Ca\(^{2+}\) signaling. The spatial segregation of Ca\(^{2+}\) signals in dendritic regions could affect the interplay of signaling mechanisms in plasticity, as shown in rat hippocampal pyramidal neurons (Nakamura et al. 2002). Furthermore, the pathway and temporal course of Ca\(^{2+}\) entry may be critical for the activation of different intracellular signal transduction processes (Gallin and Greenberg 1995). For instance, in the stomatogastric ganglion, changes in the network operation rely on the kinetics of Ca\(^{2+}\) currents (Turrigiano et al. 1995) and it has been postulated that intracellular Ca\(^{2+}\) levels affect gene expression and membrane conductance in an activity-dependant fashion (Liu et al. 1998). Perhaps long term effects of Ca\(^{2+}\) on the CPG for locomotion are altered by a similar mechanism.

In summary

Vertebrate locomotor pattern generation requires the network coordination of spinal ventral horn neurons acting in concert with their intrinsic oscillatory properties (Bianchi et al. 1995; Butt et al. 2002; Del Negro et al. 2002; Grillner et al. 2001; Marder and Thirumalai 2002). Ca\(^{2+}\) oscillations detected during NMDA-induced fictive swimming in lamprey spinal cord neurons by imaging an high affinity Ca\(^{2+}\)-sensitive dye (Oregon green 488 BAPTA 1) were correlated with fluctuations in membrane potential, and instantaneous changes in Ca\(^{2+}\) induced fluorescence coincided with spike events. Using nonratiometric dye and ratiometric measurements with Fura 2 dextran we estimated Ca\(^{2+}\) fluctuations do not exceed 1 μM. Ca\(^{2+}\) fluctuations were barely detected with dyes of lower affinity, Oregon Green 488 BAPTA 2 (k_<s>g</s> approximately 1 μM) and not at all with Fluo-4 dextran (k_<s>g</s> approximately 3 μM), providing alternative empirical evidence that Ca\(^{2+}\) responses are limited to hundreds of nm during fictive locomotion.

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