CALCIUM SIGNALING COMPONENTS OF OSCILLATING INVERTEBRATE NEURONS IN VITRO

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Abstract—We have studied the Ca\(^{2+}\) dynamics of bursting–spiking neurons in the lobster stomatogastric ganglion. Neurons in this ganglion undergo spontaneous oscillations in membrane voltage with a period of 1–10 s \textit{in situ}. We found that neurons isolated from the ganglion and filled with the fluorescent calcium indicator Fluo-4 show simultaneous changes of membrane potential and cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)). These Ca\(^{2+}\) signals are highly heterogeneous both in terms of amplitude and time constants. They showed variable spatial distributions with the soma exhibiting low and slow signals, and a region in the process with large and fast signals.

Ca\(^{2+}\) transients in the processes are dependent on external Ca\(^{2+}\) and can be blocked by Co\(^{2+}\), but not other, more specific Ca\(^{2+}\) current blockers. Rather, nifedipine a known Ca\(^{2+}\) current blocker, affects the distribution of the Ca\(^{2+}\) signal, which suggests a specific localization of Ca\(^{2+}\) channels. Although the signal is not absolutely dependent on action potentials, it is greatly reduced when action potentials are blocked by tetrodotoxin. Termination of the signal depends only slightly on Ca\(^{2+}\) buffering mechanisms such as mitochondria, Ca\(^{2+}\)/Na\(^+\) and Ca\(^{2+}\)/H\(^+\) exchangers.

We also demonstrate the presence of caffeine-sensitive internal stores in stomatogastric ganglion cells. The store distribution is different but overlaps with the voltage-dependent distribution. The maximal caffeine-activated Ca\(^{2+}\) signal is in the soma and it is smaller in the processes. Unlike the voltage-activated Ca\(^{2+}\) signal this signal is not blocked by Co\(^{2+}\). Nevertheless, the two types of signal interact during caffeine application. This unique spatial separation of two Ca\(^{2+}\) sources may have important functional implication.

Changes in free intracellular Ca\(^{2+}\) concentration have been shown to activate a plethora of cellular process in neurons including memory and neurotransmitter release (Berridge, 1998; Mattson et al., 2000). However, the role of intracellular Ca\(^{2+}\) in controlling bursting neurons is incompletely understood.

The bursts in neurons are produced by a combination of slow inward currents that produce the depolarizing phase, which in turn activate a slow outward current that terminates the burst (Calabrese, 1998; Harris-Warrick, 1993). In addition, faster voltage-gated currents are present which account for the spikes during the depolarized phase. This permits both action potentials (APs) and bursting to coexist. In this respect Ca\(^{2+}\) has been hypothesized to play a very important role as it is part of the inward current but its accumulation may also activate Ca\(^{2+}\)-activated K\(^+\) currents that have been shown to terminate bursts in several systems (Amini et al., 1999; Kiehn and Harris-Warrick, 1992). These basic principles are common in many diverse systems such as the mammalian respiratory system (Richter et al., 1992), Tritonia (Getting and Dekin, 1985) Clione swimming (Arshavsky et al., 1985) and leech heartbeat (Calabrese and Arbas, 1989). It has also been shown in what is perhaps the most extensively studied motor-pattern-generation system—the lobster stomatogastric ganglion (STG) (Gola and Selverston, 1981; Golowasch et al., 1992).

This ganglion composed of about 30 neurons, mostly motor neurons, and controls the movements of the lobster foregut (Selverston and Moulins, 1987). During normal activity, these motor neurons oscillate between a bursting and hyperpolarized state with a period of 1–10 s, depending on which of two systems they belong to (Harris-Warrick et al., 1992; Maynard and Selverston, 1975). Neurons of the pyloric system burst with a 1-s period and the gastric system neurons burst with 10-s period. The oscillations are produced in part by the network properties but mainly by the neurons which are capable of oscillating under certain conditions such as when neuromodulators (Bal et al., 1988; Selverston, 1977; Selverston and Miller, 1980) are present. With this system, it is also possible to dissociate mature identified neurons and study their properties \textit{in vitro} while they still maintain some of their properties, such as the ability to oscillate (Panchin et al., 1993).

Several types of voltage-dependent Ca\(^{2+}\) currents (VDCC) have been found in the STG (Hurley and Graubard, 1998; Turrigiano et al., 1995; Zhang and Harris-Warrick, 1995). There are at least two pharmacologically distinct Ca\(^{2+}\) currents, one located mainly in the nerve terminals and the other in the more proximal neural processes. These two Ca\(^{2+}\) currents are both blocked by Co\(^{2+}\) and Ca\(^{2+}\), but the currents in the processes are more susceptible to nicardipine and nifedipine (Hurley and Graubard, 1998). After few days in culture the isolated
STG neurons also express (Turrigiano et al., 1995) Ca\(^{2+}\) currents in the soma which were blocked by Cd\(^{2+}\) and Co\(^{2+}\). These Ca\(^{2+}\) currents could be further divided into a fast inactivating current and a persistent Ca\(^{2+}\) current that are activated at \(-40\) mV.

Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores may also play an important role in the control of cytosolic Ca\(^{2+}\) levels in STG neurons. Indeed, many hormones and neuromodulators have been shown to release Ca\(^{2+}\) from inositol-1,4,5-trisphosphate (IP\(_3\))-sensitive stores and induce changes in the neurons’ properties (Berridge, 1997). Other locations, such as ryanodine-sensitive stores, are responsible for production of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Both these stores are instrumental in the phenomena of Ca\(^{2+}\) waves observed in excitable tissue (Chen et al., 1997; Li et al., 1997) as well as non-excitatory (Li et al., 1994, 1995) cells. In these cells, cytoplasmic Ca\(^{2+}\) concentration shows marked slow (minutes period) oscillations originated mainly from internal stores. These oscillations may be triggered by external modulators (Keizer and De Young, 1992; Kojima et al., 1992) or stimuli like fertilization. Regardless, they demonstrate the importance of internal stores in the control of cytosolic Ca\(^{2+}\) levels and possible effect on ionic currents.

Using imaging techniques, it has been shown that the Ca\(^{2+}\) levels in the STG neurons follow the bursting activity of the neurons (Graubard and Ross, 1985; Ross and Graubard, 1989). They showed that there was no Ca\(^{2+}\) signal in the axon and in the soma when the neuron was bursting. However, the soma showed Ca\(^{2+}\) changes when depolarized directly. The authors raised the possibility that different mechanisms govern the Ca\(^{2+}\) changes in the soma and the processes. They also described phase shifts between the membrane potential and the Ca\(^{2+}\)-level changes, suggesting that the delay between the voltage-Ca\(^{2+}\) signal was due to the slow activation of the Ca\(^{2+}\) current and slower Ca\(^{2+}\) accumulation. In this pioneering study however, the resolution, both spatial and temporal, was insufficient to describe the Ca\(^{2+}\) signal in detail. A clear problem with in situ studies was that the elaborated processes of the STG neurons obscured each other and the emitted light was scattered. In addition due to the thickness of the ganglion only one focus plane could be reliably analyzed. To get around some of these problems Kloppenburg et al., 2000 used confocal imaging with much higher temporal and spatial resolution. They showed that in the very fine processes there was accumulation of Ca\(^{2+}\) that was probably correlated with synaptic release and modulated by dopamine. But here again they could focus only on a small portion of the neuron.

In this study, using acutely isolated neurons of the lobster STG, we were able to characterize the Ca\(^{2+}\) signal in almost the entire neuron with high temporal and spatial resolution. We found that the voltage-dependent Ca\(^{2+}\) signals have a unique bell-shape distribution. We also found that these Ca\(^{2+}\) signals depend on an external Ca\(^{2+}\) source and could be blocked pharmacologically. The imaging also revealed the existence of internal stores in STG neurons. Their distribution, although partly overlapping, was very different from the distribution of the voltage-dependent signals.

**EXPERIMENTAL PROCEDURES**

We used Caribbean spiny lobsters, *Panulirus argus*, supplied by local fishermen. The lobsters were cooled in ice to suppress nociceptive receptors and minimize suffering. The dorsal artery with the STG was removed and pinned out in Sylgard-coated Petri dish. All experiments conformed to NIH and international guidelines on the ethical use of animals. Experiments were performed on minimal number of animals.

**Cell isolation**

Cells were isolated from the desheathed STG as described previously (Cleland and Selverston, 1995; Jackel et al., 1994; Panchin et al., 1993). Briefly, ganglia were incubated in 2 mg/ml subtilisin (Sigma-Aldrich, USA) for 60–80 min and washed for 1–2 h. Cells were individually isolated with glass tools (Panchin et al., 1993, 1995) and plated in sterile uncoated polystyrene dishes (Falcon 3801; Becton Dickinson Labware, NJ, USA) with sterile *Panulirus* maintenance medium. Recordings were made after 1–7 h of incubation.

**Ca imaging**

After isolation, the neurons were impaled in the large process close to the soma by 30–70-MΩ electrodes loaded with 2 mM Fluo-4 (Molecular Probes, Eugene, OR, USA) in distilled water. A negative 5-nA current was delivered until the cell image, including processes, was clear but no significant part was filled above the saturation level of the photodetector. The dye was allowed to diffuse for 20 min until no further dye redistribution was observed. Although we paid special attention to not saturate the soma, occasionally the dye went into the soma faster then into the process. If saturated, the soma was excluded from the analysis. We used a Bio-Rad MRC 600 (Bio-Rad, UK) confocal system mounted on a Nikon inverted microscope (Nikon, Japan). An argon laser supplied the 488-nm excitation wavelength and the emitted light was low-pass filtered at 515 nm. Using a 20× 0.4-NA objective, three images were collected, averaged and stored for later analysis at approximately 1-Hz acquisition rate.

Using a custom program (Yorick), an area of interest in the image was selected and analyzed. The pixel values of the first 10 control images in the selected area were averaged to give a baseline fluorescence level (F0). This baseline was later used to divide the fluorescence change in each image to give the fluorescence ratio change (ΔF/F0).

For the analysis of the signal along the process we used a custom-made program in Matlab (Mathworks Inc., MA, USA). The image was thresholded to define the neuron’s border and a higher threshold was used to find the soma as the biggest and brightest spot. Then a line was drawn in the median of the area, starting from the soma center. The pixels along the line were filtered and used as the signal along the process. If the program-generated line was clearly out of the process midline it was corrected manually.

**Electrophysiology**

After filling the neuron with dye, the dye-filled electrode was removed and the neuron’s soma was impaled again with a 10-MΩ KCl-filled electrode. The intracellular recording and stimulation were done using Axoclamp B2 (Axon Inc., CA, USA). The data were saved to a computer using Axoscope (Axon Inc., CA, USA). An extra channel on the Axoscope program was used to record TTL signals at the beginning and end of imaging. These signals
allowed the synchronization of the intracellular recording on Axoscope and imaging that was stored on a different computer.

**Physiological and drug solutions**

Dissection and experiments were done in standard *Panulirus* saline, which had the following composition (in mmol/l): 479.1 NaCl, 12.7 KCl, 13.7 CaCl$_2$, 10.0 MgSO$_4$, 3.9 Na$_2$SO$_4$, 5.0 HEPES, and 5.0 TES, pH 7.4. *Panulirus* maintenance medium for isolated cells consisted of sterile, salt-balanced (to conform to the ionic composition and osmolarity of standard *Panulirus* saline) Leibovitz-15 medium (Sigma, Aldrich, USA) with 3 mg/ml glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B.

Benzamid (Alexis Corporation, CA, USA) was dissolved in water at stock concentration of 30 mM. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Tocris, Bristol, UK) was first dissolved in DMSO at a stock concentration of 100 mM. Nichardipine (Sigma, Aldrich, USA) was dissolved in 0.1-N HCl at 2.5 mM. Nifedipine (Alexis Corporation, CA, USA) was first dissolved in DMSO at a stock concentration of 10 mM. Ryanodine (Sigma, Aldrich, USA) was first dissolved in DMSO at a stock concentration of 10 mM. Tetrodotoxin (TTX; Alexis Corporation, CA, USA) was dissolved in distilled water at 100 mM. All drugs as stocks were kept frozen at −20 °C and dissolved in *Panulirus* saline before use. Caffeine (Sigma, Aldrich, USA) was dissolved in *Panulirus* saline at a concentration of 10 mM before use.

During experiments isolated cells were superfused continuously with *Panulirus* saline. All drugs studied were administered by bath application.

**Statistics**

The data are presented as mean±S.E. The significance level was 0.05.

**RESULTS**

**Characteristics of the voltage-dependent Ca$^{2+}$ signal in STG neurons**

Our first series of experiments were aimed at measuring changes in cytoplasmic Ca$^{2+}$ concentration in different parts of the isolated neuron. To do that we isolated and plated STG cells on a dish. After at least 1 h recovery, we chose healthy neurons ($n=54$) for the experiment based on their resting potentials and ability to produce action potentials (APs) either at rest or with a small injection of depolarizing current. The neurons we chose had relatively long processes that were attached to the dish. The average resting potential was −46±1 mV (similar to neurons *in situ*) and most neurons ($51/54$) were spiking at rest at 11±1 Hz. Eleven of the neurons in addition to APs firing also showed slower events, such as frequency modulation, but only one showed actual bursting under control conditions and eight showed bursting with current injection or pharmacological agents. As these neurons usually burst at a 1–10-s period and could have been subject to a higher transient Ca$^{2+}$ load than regularly spiking neurons, we used a stimulation protocol that produces activity similar to the natural neurons activity. Specifically, we hyperpolarized (with approximately 1 nA current injections) the neuron until the spiking stopped, then the neuron was released from the hyperpolarization or the current was reversed to produce depolarization and after 20 s hyperpolarized again. In response to these stimulation protocols, we refer to as, current-relaxation and depolarization respectively; the neurons usually started spiking at 18±1 Hz ($n=54$) and 34±2 Hz ($n=54$), respectively, and stopped spiking when the hyperpolarization resumed. The Ca$^{2+}$ signal increased with the depolarization until reaching a steady state and then recovered to baseline following the hyperpolarization renewal (Fig. 1). Upon hyperpolarization and termination of spiking, there was a decrease in the calcium signal, which probably meant that there was a continuous influx of Ca$^{2+}$ at rest.

From Fig. 1 it is evident that the global Ca$^{2+}$-level dynamics were much slower than the membrane-potential dynamics. Although it has been shown previously (Benham et al., 1992; Helmchen et al., 1999; Ivanov and Calabrese, 2000), the slower dynamics of the Ca$^{2+}$ may have important consequences for oscillating neurons. The slower dynamics produced a delay from the time of voltage change to the appearance of the global Ca$^{2+}$ change and Ca$^{2+}$ buildup during consecutive stimulations. In part this may be a result of the dye properties, as will be discussed later but it also reflects the response dynamics (see also Marchi et al., 2000). A key observation was that the signal was not evenly distributed in the neuron, the signal in the soma being smaller and slower than in the process (see Fig. 1).

**Distribution of the voltage-dependent signal**

We further analyzed the voltage-dependent Ca$^{2+}$ signal along different parts of the neuron. The Ca$^{2+}$ signal was relatively weak in the small branches of the processes but very pronounced in the main process. Therefore, we analyzed the signal along the main process as a function of distance from the soma (see Experimental Procedures). The histogram shown in Fig. 2A demonstrates that the signal amplitude has a bell-shape or “peaked” distribution with a region in the process having the maximal response that gradually decreases proximally to the soma and distally, into the axon. This distribution was repeated for a large majority of neurons we tested ($n=54$) and could not be attributed to a particular morphological cell type or property.

In Table 1 we compare the signal in this “peak” region to the signal in the soma. In the soma ($n=38$, range 11–727% ∆F/F0) the signal was 78% smaller in amplitude than in the “peak” ($n=54$, range 2–162% ∆F/F0). Despite the large variability between different cells, the amplitude was significantly different when trials were compared within the same cell ($P<0.01$ paired Student’s t-test). The Ca$^{2+}$ signal in the “peak” region was also 2.4 times faster in its rise time and 1.7 times faster in its decay time (Table 1), which was also significantly different ($P<0.01$, paired Student’s t-test).

In cases where we analyzed the regions between the peak and the soma (as shown in Fig. 1), values were intermediate but without proper definition of this region the variability was too large to be presented systematically.
**APs’ contribution to the Ca$$^{2+}$$ signal**

The Ca$$^{2+}$$ signal in our experiments clearly follows the firing of the neuron. To study the contribution of APs to the Ca$$^{2+}$$ signal, we repeated the stimulation protocol in the presence of 1-μM TTX (Fig. 2C).

In the presence of TTX with APs blocked (Fig. 2C), the Ca$$^{2+}$$ signal still followed the depolarization of the neuron although its amplitude was greatly reduced at the “peak” by 67±13% (n=4, and compare Fig. 2B, C). What proportion of the effect was due to the lower depolarization and what was due to the APs themselves is difficult to determine. As we analyzed the Ca$$^{2+}$$ signal along the process we could see an interesting result. The “peaky” distribution of the Ca$$^{2+}$$ signal was flattened when the APs were blocked (Fig. 2C). This means that the effect of the AP block was stronger at the “peak” region than in the other regions. In fact in the four experiments performed, the reduction in amplitude was significant in the “peak” (P<0.01) but not in the soma region (P>0.4).

Regardless of the mechanism (see Discussion), the “peak” region signal was mostly AP activated, whereas, Ca$$^{2+}$$ levels in the soma were much less sensitive to APs.

This could also be seen by plotting the Ca$$^{2+}$$ signal against AP frequency which yielded a linear slope value for the individual experiment. The average slope for the soma was 0.015±0.002 (n=38) whereas it was 0.071±0.007 (n=58) in the “peak.” The location is not easy to characterize, as the morphology of the neuron is lost when it is extracted from the ganglion but in all neurons studied the Ca$$^{2+}$$ signal started going up several tens of microns away from the soma and covered the region where the fine processes come out of the main process. The “peak” is not associated with specific structures such as branch points or varicosities; rather, it is located in the middle of the region that shows the Ca$$^{2+}$$ signal. The fact that TTX flattened the Ca$$^{2+}$$ signal distribution or in other words removed the peak, suggests that the high concentration of Na$$^{2+}$$ channels is co-localized.

Further indication for the differential sensitivity of the region appeared when looking at the ratio between the Ca$$^{2+}$$ signal in soma and in the “peak” (soma to peak ratio). On average this ratio was 28±4 (n=38) in the current relaxation experiment and 33±5 (n=38) in the depolarization experiment. This small difference was
despite the fact that the frequency of the AP almost doubled.

**Dependence of the Ca^{2+} signal on external Ca^{2+}**

To investigate whether Ca^{2+} influx was required for the activation of Ca^{2+} during bursts we used a Ca^{2+}-free saline. Unfortunately, the STG neurons do not tolerate nominally Ca^{2+}-free solution well and usually did not continue firing for more than 10 min. However, in two of the successful Ca^{2+}-free experiments (n=5), where the neurons were spiking at rest they depolarized and continued firing, upon removal of Ca^{2+} from the solution. The rest of the neurons (n=3) that were not firing at rest, showed no change in resting potential. The resting Ca^{2+} level dropped markedly in all tested neurons, following a transient sharp rise (data not shown). Using the current relaxation protocol, 92±4% (n=5) of the Ca^{2+} response in the “peak” region was blocked (as compared with that in control; Fig. 3). This indicates that the Ca^{2+} signal, that we recorded, depends on external Ca^{2+}, if not for the entire response, at least for its triggering.

To determine the extent to which external Ca^{2+} contributed, we blocked Ca^{2+} influx by Co^{2+} (20 mM), a concentration routinely used to block Ca^{2+} currents in STG neurons (Zhang and Harris-Warrick, 1995; Zhang et al., 1995). In all cells studied (N=12) Co^{2+} bath application, induced depolarization (9±1 mV) followed by an increase in AP frequency (1.7 times, except for two cells that were not spiking before the Co^{2+} and two cells that started to oscillate, which made frequency comparison impossible.) In seven of nine cells, depolarization reached a sustained plateau at which cells stopped spiking. The remaining two cells continued to exhibit APs but the frequency was reduced to about twice that of the controls.

As in the case of the Ca^{2+}-free solution, application of Co^{2+} caused a sharp drop in the resting Ca^{2+} level (50±6 and 28±7%, and in the “peak” and soma regions, respectively). In several cases a transient sharp Ca^{2+} rise preceded the drop.

Metal ions including Co^{2+} have been shown to affect Ca^{2+} indicator properties. In vitro testing that we did with the dye showed up to a 30% reduction of fluorescence with 20-mM Co^{2+}, which may explain most of the changes in resting Ca^{2+} level, but the Ca-dependent fluorescence change was not affected as much (see also Marchi et al., 2000) and as we were interested mainly in changes induced by voltage, we could still use Co^{2+}. It is important to note that in the presence of Co^{2+}, all cells studied continued to respond to current relaxation by APs, although in nine of 12 cells spiking during current relaxation was transient.

Under these conditions the Ca^{2+} signal induced by current relaxation was almost completely absent. On average the Ca^{2+} signal was reduced by 88±3% (n=12) and by 79±10% (n=7) in the “peak” region and the soma, respectively.
The effect of specific Ca$^{2+}$/H11545 current blockers

Although, Cd$^{2+}$ (100 M) has been widely used in the STG, as a general Ca$^{2+}$/H11001 current blocker, it only blocked about 30% of the Ca$^{2+}$/H11001 signal. It also had some toxic effect on STG neurons; we therefore tried more specific Ca$^{2+}$/H11001 channel blockers to examine the contribution of voltage dependent calcium current (VDCC) to the transient Ca$^{2+}$/H11001 signal in STG neurons. In mammals, nifedipine and nicardipine are specific L-type Ca$^{2+}$/H11001 channel blockers, which have also been shown to block VDCC in the STG (Hurley and Graubard, 1998). As in the case of Cd$^{2+}$/H11001, the drugs did not eliminate the signal completely (Fig. 4). Although it was difficult to quantify the signal amplitude change because of the drugs’ effect on neuron spiking, there was clear reduction of Ca$^{2+}$/H11001 response amplitude: in the “peak” region the drugs blocked 62±11% (n=5) of the response but only 43±13% (n=4) in the soma. The soma to “peak” ratio, as in the case of TTX, was significantly reduced after the drug application (n=7, P<0.01, paired Student’s t-test).

Interestingly, when examining the signal distribution along the process one can see that the “peak” distribution of the signal exists in the drugs’ presence. However, in the distal part of the process, the signal was reduced (Fig. 4) which resulted in a drop-off of the Ca$^{2+}$/H11001 signal distally. Similar results were seen in five other neurons.

Internal stores contribution to the Ca$^{2+}$/H11545 signal

Although much of the Ca$^{2+}$/H11545 signal comes from external sources, internal Ca$^{2+}$/H11001 stores may also make a contribution. To demonstrate the existence of internal stores in the STG we bath applied 10-mM caffeine to the isolated neurons. Caffeine has previously been shown to have a physiological effect on STG neurons (Hermann, 1981). Upon application, the neurons showed a depolarization of 13±3 mV (n=5) and transient approximately 60% increase in the Ca$^{2+}$/H11001 signal. The Ca$^{2+}$/H11001 change had a much slower time course than for current injection, probably reflecting a property of the application system.

Unlike the “peaky” distribution of the Ca$^{2+}$/H11545 signal induced by current relaxation, with caffeine application the signal was slightly more pronounced closer to the soma (data not shown). This could be measured quantitatively by comparing the relative caffeine-induced Ca$^{2+}$/H11001 signals in the soma and “peak” to the increase induced by depolarization (Fig. 5A). If this increase only reflected the voltage-activated entry due to depolarization, these relative Ca$^{2+}$/H11001 signals should be equal. However, it was higher in the soma (P<0.01, n=10), indicating that the Ca$^{2+}$/H11001 increase was higher here than what would be expected by a change of membrane potential alone.

Table 1. Parameters of Ca signal induced by voltage and 10 mM caffeine in isolated lobster neurons

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<tr>
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<th>Ca signal induced by current relaxation</th>
<th>Ca signal induced by depolarization</th>
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<tbody>
<tr>
<td>ΔF/F₀, %</td>
<td>Rise-time constant, s</td>
<td>Decay-time constant, s</td>
</tr>
<tr>
<td>Peak</td>
<td>179±14 (57)</td>
<td>3.4±0.2 (63)</td>
</tr>
<tr>
<td>Soma</td>
<td>40±5 (38)*</td>
<td>8.2±0.9 (34)*</td>
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* Differences between parameters induced by current relaxation or depolarization in the peak and soma are significant.
† Difference between rise-time constant induced by current relaxation and depolarization in the soma is significant.

Mean±S.E.

Fig. 3. Simultaneous intracellular recording (bottom traces) and Ca$^{2+}$/H11001 signal (top traces) on a neuron exposed to Ca$^{2+}$/H11001-free solution. The bars indicate the stimulus protocol; white, hyperpolarization; black, depolarization.
The nature of this depolarization could be Ca\(^{2+}\) ion flux from internal stores into the cytoplasm but it also could be accompanied by ionic currents. These currents could be capacitive Ca\(^{2+}\) currents triggered by the emptying of internal stores (Berridge, 1995; Dutta, 2000) or other currents activated by a Ca\(^{2+}\) increase. To further investigate these possibilities in a more direct way, we applied caffeine in the presence of Co\(^{2+}\). As 20-mM Co\(^{2+}\) almost completely blocks the voltage-dependent signal (see also Fig. 5C) we did not expect a significant

Fig. 4. Ca\(^{2+}\) signal changes along the main process analyzed as in Fig. 2. (A) Current relaxation protocol in control conditions. (B) Same protocol applied in the presence of 10-μM nicardipine. Note that the distal part is reduced after nicardipine. Subtraction of B from A is shown in C. The bars on the time axis indicate the stimulus protocol; white, hyperpolarization; orange, depolarization.
Fig. 5. Ca$^{2+}$ changes induced by caffeine. (A) The relative Ca$^{2+}$ change induced by caffeine to the change induced by current relaxation protocol in the soma and the "peak" region of the process. (B) Voltage-induced changes in Ca$^{2+}$ signal as in Fig. 2 but with absolute change in ΔF/F0. (C) Same protocol as in B, in the presents of Co$^{2+}$ shows very small Ca$^{2+}$ increase in a limited area. The signal is normalized to emphasize the distribution. The bars on the time axis indicate the stimulus protocol; white, hyperpolarization; orange, depolarization. (D) Application of caffeine in the presence of Co$^{2+}$ shows increase closer to the soma. Axes as in C but the time course is longer. Start of caffeine application indicated by the orange bar.
contribution from Ca$^{2+}$ currents or nonspecific cationic currents.

In the group of neurons tested under the above conditions we could draw a distinction between neurons that were firing APs in the presence of Co$^{2+}$ and those that were quiescent. In the active neurons (five of nine), caffeine caused a depolarization of 12±6 mV. In addition the Ca$^{2+}$ level increased as can be seen in a representative example, Fig. 5D. The neurons that stopped firing APs in Co$^{2+}$ ($n = 4$) showed no change in membrane potential or Ca$^{2+}$ level after caffeine application. This suggests that some kind of Ca$^{2+}$ entry is necessary for the caffeine-induced signal to exist. Perhaps without APs even the smallest amount of Ca$^{2+}$ could not go in, which in turn depleted the internal stores so that they would not respond to caffeine.

We received further support for this possibility with caffeine application in Ca$^{2+}$-free saline. Here the neurons stopped spiking after few minutes in Ca$^{2+}$-free media and caffeine failed to induce any change in resting potential or in the Ca$^{2+}$ signal. This would be expected if the internal stores were depleted and could not be renewed from extracellular sources.

When analyzing the Ca$^{2+}$ signal along the process with Co$^{2+}$ present (Fig. 5D) the pattern was similar although more pronounced than the pattern with caffeine application alone. It grew larger nearer to the soma where it was the biggest. Furthermore, it was consistent with the fact that the voltage-activated channels were blocked and most of the signals we recorded were the result of release from internal stores that are located close to the soma.

We also tried to see the contribution of these caffeine-sensitive stores to the signal induced by long depolarization or current relaxation as in Fig. 1. We assumed that a long application of caffeine would deplete the stores and allow us to measure the difference. Using the same protocol with pre-incubation of 20–30 min in 20-mM caffeine gave somewhat surprising results. The signal in the peak region decreased by 44±9% but in the soma the signal increased significantly ($P < 0.05$) by 58±32% ($n = 4$). One possible explanation for this result was the mixed effect of caffeine. On the one hand it depleted internal stores but on the other it also elevated Ca$^{2+}$ levels especially in the soma, which in turn may sensitize channels hence, increasing Ca$^{2+}$ currents.

The complementary experiment of blocking the stores with 30-$\mu$M ryanodine did not significantly change the amplitude or the time constants of the Ca$^{2+}$ signal. It also had no apparent effect on the resting potential or the AP frequency of the neurons.

**Contribution to the Ca$^{2+}$ signal from other sources**

Other mechanisms have been shown to participate in Ca$^{2+}$ buffering, especially in neurons with a high Ca$^{2+}$ load. We specifically tested the mitochondrial contribution to Ca$^{2+}$ removal and the contribution of Ca$^{2+}$/Na$^+$ and Ca$^{2+}$/H$^+$ exchangers both in its reverse-mode activity to the Ca$^{2+}$ signal during depolarization, and the removal of Ca$^{2+}$ at the end of the depolarization.

We used two methods to assess the contribution of the Ca$^{2+}$/Na$^+$ exchanger. First we substituted Na$^+$ with Li$^+$ (Fig. 6A), which could not operate the exchanger. In this condition applying the current relaxation protocol resulted in no change in the signal amplitude ($94±15\%$ of the control level, $n = 4$) and no change in the rise time constant ($3.26±0.99\ s$ and $4.31±1.63\ s$, in control and Na$^+$-free solution, respectively). However, there was a significant ($P < 0.05$) increase in the decay time constant from $6.77±1.99\ s$ under control conditions to $10.89±3.39\ s$ in Na$^+$-free solution (Fig. 6A). Similarly, in two experiments where we blocked the exchanger with 100-$\mu$M benzamil there was no change in the amplitude or rise-time constant and some increase (from $3.51±0.52\ s$ to $5.94±0.18\ s$) in the decay-time constant. Thus the exchanger does not elicit the main Ca$^{2+}$ increase and does not change the amplitude of the signal at the steady state but contributes to the Ca$^{2+}$ removal.

We also blocked the Ca$^{2+}$/H$^+$ exchanger by alkaline (pH = 8.4) solution (Fig. 6B). This was expected to reduce Ca$^{2+}$ removal and if it was activated during the depolarized phase should increase the amplitude. Instead, as in the case of the Ca$^{2+}$/Na$^+$ exchanger, it only changed the decay-time constant from $6.24±1.71\ s$ to $9.45±1.09\ s$ ($n = 4$). This suggests that these mechanisms are not significantly activated during the time of depolarization but have some role in the decay phase at the depolarization end.

Another possible mechanism that we tested is mitochondrial uptake, which has been shown to remove Ca$^{2+}$ at high concentration (Duchen, 1999; Murchison and Griffith, 2000). We used 10-$\mu$M CCCP to stop mitochondrial activity and examined the effect on the Ca$^{2+}$ signal using the current relaxation protocol. If the mitochondria were taking part in the Ca$^{2+}$ removal during the depolarization phase, we would expect the signal to increase with the mitochondrial blockage. Instead in all the experiments ($n = 4$) the signal amplitude was reduced (data not shown) there was no significant change in the rise or decay time. This result may be attributed to some metabolic change in the neurons due to ATP shortage and not the direct effect of the mitochondria on Ca$^{2+}$ removal.

Our conclusion is that although these mechanisms may participate in the Ca$^{2+}$ signal during depolarization they are neither the main contributors nor crucial to the Ca$^{2+}$ signal.

**DISCUSSION**

It is not surprising that channels and thus currents are not evenly distributed in neurons. Different neuron regions have different functions and properties; therefore, Ca$^{2+}$ signals as well should be spatially different (Pozzo-Miller et al., 2000; Seymour-Laurent and Barish, 1995) depending on their role in neuron function. The challenge is to investigate how the spatially heterogeneous Ca$^{2+}$ signals are coordinated. However, to do that we first need to understand the mechanisms that control these Ca$^{2+}$ signals. In
In this study we provide evidence supporting the spatial separation of two sources of Ca\(^{2+}\) that have to be coordinated under natural conditions. The heterogeneous Ca\(^{2+}\) signal in the STG neurons has a maximum in the local part of the process that decays toward the axon and the soma. The Ca\(^{2+}\) entry is not from the APs themselves (in the axon), rather Ca\(^{2+}\) channels, exist that are mostly responsible for Ca\(^{2+}\) entry. Multiple mechanisms of intracellular Ca\(^{2+}\) elevation together with their specialized distribution in different cell compartments allow STG neurons to perform their complex behavior.

As suggested in previous studies (Ross and Graubard, 1989) and by the present results, STG neurons possess multiple mechanisms of Ca\(^{2+}\) signaling which we tried to characterize. There is a significant VDCC activated probably during AP generation and blocked by specific Ca\(^{2+}\) channel blockers such as nicardipine and nimodipine. In addition there are low-threshold Ca\(^{2+}\) and/or nonselective cationic channels blocked by Co\(^{2+}\) but not by any known specific current blocker. Unlike the previous results that only described external Ca\(^{2+}\) entry (Ross and Graubard, 1989) we could demonstrate caffeine-sensitive internal Ca\(^{2+}\) stores. And finally Ca\(^{2+}\) extrusion through Ca\(^{2+}\)/Na\(^{+}\) and Ca\(^{2+}\)/H\(^{+}\) exchangers plays a role in terminating Ca\(^{2+}\) elevation but not so much in the Ca\(^{2+}\) entry itself. External Ca\(^{2+}\) plays a crucial role not only because it enters the neuron through ionic channels in the membrane, but also seems to be necessary for the triggering of Ca\(^{2+}\) release from internal sources and for the subsequent refilling. The latter mechanism is probably responsible for the deteriorating effect of Ca\(^{2+}\)-free saline on STG neurons observed in our experiments.

Fig. 6. Simultaneous intracellular recording (bottom traces) and Ca\(^{2+}\) signal (top traces). In A the current relaxation protocol was applied, in control condition, after replacement of Na\(^{+}\) with Li\(^{+}\) and after Na\(^{+}\) reintroduction. (B) Same as A, but with external pH rise to 8.4. The bars indicate the stimulus protocol; white, hyperpolarization; black, resting potential.
Our results support previous studies showing that the Ca\textsuperscript{2+} signal is absent in the axon and is smaller in the soma of STG neurons (Graubard and Ross, 1985; Ross and Graubard, 1989). The previous studies used slow Ca\textsuperscript{2+} dye (arsenazo III) with low sensitivity to Ca\textsuperscript{2+} level changes; therefore they had to use excessive averaging. With our method we had much better temporal and spatial resolution and could systematically characterize the Ca\textsuperscript{2+} signal in these neurons finding that the distribution of the Ca\textsuperscript{2+} signal along the process, has local peaks. By using isolated neurons almost the entire neuron was in the same focal plane of the microscope and we had no problem with light scattering and processes overlap. In addition the neurons in our experiments were more electrically compact allowing for better spread of voltage along the membrane. As the neurons in our study were physically isolated, we were not concerned about indirect effects from the network such as synaptic input, so that all of the signal measured was intrinsic to the cell.

A clear drawback in studying neurons in vitro is that taken out from their natural environment the neurons may show modified properties. For instance, ionic currents were studied in isolated STG soma with re-grown process (Turrigiano and Marder, 1993). It is important to note that, STG neurons change after the first day in culture and new properties appear (Panchin et al., 1993). Nonetheless, as we used the neurons soon after isolation we avoided the problem of long-term changes. Although, it is possible that the isolation procedure somewhat damaged the neurons. For instance, pulling out the neurons probably tore out the very fine processes, the same processes that local Ca\textsuperscript{2+} release is suggested to be a result of neuronal activity. In addition, the dendritic spines or any other distal processes may be damaged. Hence, other factors may contribute to the observed changes. For instance, with Ca\textsuperscript{2+} current blocking we only found partial blocking of Ca\textsuperscript{2+} signal. To figure out what other currents participate in the Ca\textsuperscript{2+} signal awaits more specific pharmacology of the different channels.

The fact that specific Ca\textsuperscript{2+} current blockers modify the distribution shape indicates that at least some of the Ca\textsuperscript{2+} channels are localized, but the change of the signal indicates that some VDCC are not located at the "peak" region itself; rather, they are more distal. Hence, other factors determine the single unique distribution of Ca\textsuperscript{2+} signal in these neurons. These factors may include Na\textsuperscript{+} channels in the spike initiation zone or other unknown Ca\textsuperscript{2+} permeable channels. A definitive answer to this question cannot be given without clear immunocytochemistry. Recently localized distribution of voltage-gated Ca\textsuperscript{2+} channels in the neuropile of the STG has been demonstrated using Ca\textsuperscript{2+} imaging (Kloppenburg et al., 2000) and specific antibodies to the partial clone of lobster Ca\textsuperscript{2+} channel cDNA (French et al., 2000). Although these sites probably coincide with the sites of neurotransmitter release and are different from those analyzed here, the data clearly demonstrate an uneven distribution of ionic channels in the membrane of STG neurons. In addition, (Baro et al., 2000) demonstrated specific localization of different potassium channels.

In a good agreement with a previous study of Ca\textsuperscript{2+} signals in STG neurons we demonstrate the membrane-potential changes induce a prominent Ca\textsuperscript{2+} signal in the soma. In previous experiments on isolated neurons (Panchin et al., 1993; Turrigiano and Marder, 1993) it was shown that the acutely isolated cells do not possess significant inward currents in the soma. It was confirmed in our experiments using whole-cell patch-clamp recording from freshly isolated STG neuron soma (M. Samoilova, unpublished observations). No inward currents are detected in responses to depolarizing holding potentials up to +30
The absence of Ca\(^{2+}\) current in isolated STG neuron soma can result from specific properties of Ca\(^{2+}\) channels. Ca\(^{2+}\) channels could be silent under resting condition, being under inhibitory control of some unknown factor(s) or requiring some factor(s) to become active. These factors could be controlled by backpropagating APs. Recently the existence of such silent Ca\(^{2+}\) current has been revealed in crustacean muscles (Enxleben and Hermann, 2001; Monterrubio et al., 2000). The hypothesis is not supported by the absence in STG neuron soma of immunolabeling to specific lobster Ca\(^{2+}\) channel antibodies (French et al., 2000).

Simple diffusion of free Ca\(^{2+}\) from the site of Ca\(^{2+}\) entry in the process toward the soma cannot account for the Ca\(^{2+}\) signal induced by voltage in the soma. There is virtually no time delay between the signal in the process as far as 400 µm and the soma. Even faster imaging techniques that allowed 16-ms temporal resolution (data not shown) did not reveal any time delay. The question arises then what could be the nature of this signal? It is known that the initiation of a global Ca\(^{2+}\) signal by external stimuli depends on the progressive recruitment of small local events. “Ca\(^{2+}\) puffs,” and involves intracellular Ca\(^{2+}\) stores (for review see Berthoud, 1995, 1997; Mattson et al., 2000; Thomas et al., 2000). Thus, local increases of Ca\(^{2+}\) concentration by influx of extracellular Ca\(^{2+}\) through plasma-membrane Ca\(^{2+}\) channels can induce local Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores (through inositol trisphosphate (IP\(_3\)) and ryanodine receptors) in close vicinity of the Ca\(^{2+}\) entry site. This local Ca\(^{2+}\) release can in turn induce Ca\(^{2+}\) release from neighboring sites, amplifying the initial release into a Ca\(^{2+}\) wave. These events result in the appearance of Ca\(^{2+}\) responses in the cell region remote from the initial site of Ca\(^{2+}\) entry. The existence of a similar process in STG neurons remains to be elucidated. Unfortunately, there are no data on the presence of IP\(_3\) and ryanodine receptors in STG neurons but a morphological basis for their localization—endoplasmic reticulum—has been shown (King, 1976) to be located mainly in the soma and primary process. The lack of ryanodine effect in our experiments does not necessarily mean that Ca\(^{2+}\)--induced Ca\(^{2+}\) responses do not exist in these cells, rather the pharmacology may be different, as is the case with many invertebrate systems.

In order to explain the functional significance of the unique Ca\(^{2+}\) signal distribution it is important to relate it to the spike initiation zone of these neurons. It is thought that the spike initiation zone is located in the posterior part of the ganglion (Raper, 1979) just where the fine processes end. This, in general, corresponds with the location of the Ca\(^{2+}\) signal in our experiments. A further indication for the association of the spike initiation zone and the Ca\(^{2+}\) signal comes from the fact that eliminating the APs resulted in a changed Ca\(^{2+}\) distribution. This AP-dependent Ca\(^{2+}\) signal distribution may result from the fact that when APs are blocked the local membrane potential is reduced, therefore VDCC will be less activated. However, the unique Ca\(^{2+}\) distribution that we found could be created in two ways. The high-threshold VDCCs are distributed evenly along the process but the spike initiation zone with a high concentration of Na\(^{+}\) channels is in the “peak” region. The other possibility is that the “peak” represents a hot spot of high-threshold VDCC. The latter means that blocking the VDCC should result in a greater effect on the peak region than in the soma. Regardless of the mechanism the fact is that the “peak” of the Ca\(^{2+}\) signal and spike initiation zone are co-localized.

The implication of this co-localization is that the spike-producing currents and the Ca\(^{2+}\) signal are not delayed by distance and therefore may operate on the same time scale. In theoretical studies it was shown that the different time scales of spikes and internal Ca\(^{2+}\) levels are crucial for the regular rhythmic activity of STG neurons (Falke et al., 2000; Li and Rinzel, 1994; Varona et al., 2001). In this model the internal Ca\(^{2+}\) levels change slowly (due to IP3 modulation) which produces irregular bursting in isolated neurons. These irregularities disappear when the neurons are connected in the network. A precondition for such a result is separation in time between the processes. As the VDCC are not separated an alternative candidate could be the caffeine-sensitive stores, which we have shown to be located away from the “peak” of the Ca\(^{2+}\) signal; therefore, the response of this store to Ca\(^{2+}\) elevation would be delayed.

An additional functional implication is that the Ca\(^{2+}\) signal amplitude in the “peak” depends linearly on AP frequency, without saturation. But the fact that cell hyperpolarization usually results in a decrease in the Ca\(^{2+}\) signal suggests that there is a continuous influx of Ca\(^{2+}\) into the cell in resting non-spiking neurons. As the neurons in our experiments were fairly close to the normal resting potential this is likely to occur in the intact ganglion as well. In fact, in preliminary experiments on neurons in situ, we see similar results (R. Levi, unpublished observations). In other words the cytoplasmatic Ca\(^{2+}\) level monitors the neuron voltage level. In hyperpolarized neurons the Ca\(^{2+}\) level is low, it is higher in tonically spiking neurons and is alternating at levels higher than rest, in bursting neurons. Because the time constants of the voltage-induced Ca\(^{2+}\) signal are slower than the bursting period it will accumulate until reaching a steady state depending on the bursting parameters (such as period AP frequency and duty cycle), and the particular Ca\(^{2+}\) buffering of the individual neuron.

In this study we characterized the spatial distribution of Ca\(^{2+}\) signals from different sources in bursting neurons in the STG. Although these results may have significant consequences for the function of the system it would be difficult to demonstrate this in isolated cells. Rather these pharmacological tools have to be used in more intact preparations in order to reveal the functional significance of different Ca\(^{2+}\) sources and dynamics.

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