Initiation and Propagation of Calcium-Dependent Action Potentials in a Coupled Network of Olfactory Interneurons

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Received 24 May 2000; accepted in final form 18 October 2000

Wang, Jing W., Winfried Denk, Jorge Flores, and Alan Gelperin. Initiation and propagation of calcium-dependent action potentials in a coupled network of olfactory interneurons. J Neurophysiol 85: 977–985, 2001. Coherent oscillatory electrical activity and apical-basal wave propagation have been described previously in the procebral (PC) lobe, an olfactory center of the terrestrial slug Limax maximus. In this study, we investigate the physiological basis of oscillatory activity and wave propagation in the PC lobe. Calcium green dextran was locally deposited in the PC lobe; this led to cellular uptake and transport of dye by bursting and nonbursting neurons of the PC lobe. The change of intracellular calcium concentration was measured at several different positions in neurites of individual bursting neurons in the PC lobe with a two-photon laser-scanning microscope. Fluorescence measurements were also made from neurons intracellularly injected with calcium green-1. Two different morphological classes of bursting neurons were found, varicose (VB) and smooth (SB). Our results from concurrent optical and intracellular recordings suggest that Ca$^{2+}$ is the major carrier for the inward current during action potentials of bursting neurons. Intracellular recordings from bursting neurons with nystatin perforated-patch electrodes made while simultaneously recording the local field potential (LFP) with extracellular electrodes indicate that the burster spikes are precisely phase-locked to the periodic LFP signals. By referencing successive calcium measurements to the common LFP signal, we could therefore accurately determine the relative timing of calcium transients at different points along a neurite. Measuring the relation of temporal to spatial differences allowed us to estimate the velocity of action potential propagation, which was 4.3 ± 0.2 (SE) mm/s in VBs, and 1.3 ± 0.2 mm/s in SB.

INTRODUCTION

Synchronous firing of cortical principal neurons resulting from coupled inhibitory neuron activity may be the basis for feature binding and pattern recognition (Gray 1999; Hopfield 1999; Singer 1999). Progress has been made in understanding how coupled inhibitory networks can promote synchrony (Galarreta and Hestrin 1999; Gibson et al. 1999). However, the mechanism by which action potential (AP) propagation contributes to the properties of inhibitory networks is not well understood. We therefore studied this problem using a network of coupled inhibitory interneurons in the olfactory system of the terrestrial mollusk Limax maximus.

The highly developed olfactory system of Limax is accessible to physiological recordings and optical imaging (Delaney et al. 1994; Gelperin et al. 1996; Kleinfeld et al. 1994). It provides a model system to study olfactory information processing and learning (Gelperin 1999). Olfaction is the most important sense for Limax, which has no auditory system and only a very primitive visual system. A significant portion of the CNS is devoted to processing olfactory information. The procebral (PC) lobe, which is the olfactory processing center, displays waves of cellular excitation that propagate from the apex to the basal region of the PC lobe as shown by field potential recordings and optical imaging with voltage-sensitive fluorescent dye (Delaney et al. 1994; Gelperin and Tank 1990; Kawahara et al. 1997; Kleinfeld et al. 1994). The oscillatory activity is dependent on endogenous nitric oxide (Gelperin 1994), modified by odor stimulation (Delaney et al. 1994; Gervais et al. 1996; Kimura et al. 1998; Kleinfeld et al. 1994) and modulated by endogenous neurotransmitters such as dopamine, serotonin, and glutamate (Gelperin 1999; Gelperin et al. 1993). Oscillatory activity in the PC lobe is required for discriminating between closely related odor molecules in an isolated nose-brain preparation (Teyke and Gelperin 1998). The PC lobe contains two cell types, classified as “bursting” and “nonbursting,” based on their physiological (Kleinfeld et al. 1994) and morphological (Watanabe et al. 1998) properties. Based on these data, a minimal model of waves and oscillations in the PC lobe has been proposed (Ermentrout et al. 1998).

A two-photon laser-scanning microscope (2PLSM), having the advantages of reduced phototoxicity and deeper tissue penetration over confocal or conventional fluorescence microscopy (Denk and Svoboda 1997; Denk et al. 1990), is suitable for measuring intracellular calcium dynamics of PC cells (Gelperin et al. 1996), which lie in a layer more than 100 μm in thickness adjacent to a dense neuropil region. In this report, we present results from simultaneous intracellular recording and [Ca$^{2+}$] imaging of bursting neurons that suggest that APs in these cells are mediated in large part by Ca$^{2+}$ ions. By making use of a common local field potential (LFP) signal as a reference, we were able to temporally align optical recordings that were made at different times from multiple points along a burster cell neurite. This allowed us to deduce Ca$^{2+}$ AP arrival times at different locations and thus determine the initiation point(s) and the conduction velocities of APs along a neurite. Two distinct classes of bursting neurons were found, clearly separated by their conduction velocity and morphology.

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METHODS

Animals (Limax maximus) were reared at 16°C with ad libitum access to lab chow (Purina) supplemented with vitamins, sea sand, and the fungicide Tegosept (methyl-p-hydroxybenzoate). PC lobes were isolated from the cerebral ganglia by microdissection. During dissection, synaptic activity was reduced by a sixfold increase of Mg2+ concentration to 27.6 mM with Na+ concentration reduced accordingly to maintain the same osmotic pressure. Before the start of recording, desheathed PC lobes were embedded in 1–2% low gelling temperature agarose (A-0701, Sigma, St. Louis, MO) in saline <38°C with the cell layer of the PC lobe facing upward. Saline was subsequently added to cover the agarose gel for physiological recordings. Limax saline contained (in mM) 55.4 Na+, 4.2 K+, 7.0 Ca2+, 4.6 Mg2+, 80.1 Cl−, 0.2 H2PO4−, 2.5 HCO3−, 5.0 glucose, and 10 HEPES buffered to pH 7.6 (Delaney and Gelperin 1990). Isolated PC lobes were used for experiments within 24 h.

The following procedure is used to label the PC neurons with calcium green 10k-dextran (CGD, Molecular Probes, Eugene, OR) (Gelperin and Flores 1997). A paste of CGD was made with distilled water on a depression slide. The tip of a glass electrode was dipped into the paste so that a small amount of dye adhered to the tip. The dye-coated electrode was subsequently dried for 1 h in a stream of warm air. The bulk solution of saline surrounding a PC lobe was removed, and the dye-coated electrode was used to stab the PC lobe and deposit dye densely in a restricted area of the cell layer. Fresh saline was added after dye application. Cells were well labeled 6–12 h after dye application.

For intracellular injection of calcium green-1 (CG-1, Molecular Probes), electrodes were fabricated from quartz capillary glass (1.0 mm OD and 0.5 mm ID, Sutter Instrument, Novada, CA) and pulled with a laser puller (P-2000, Sutter Instrument). Parameters of the laser puller were: heat, 905; filament, 5; velocity, 50; delay, 140; pull, 175. Resistance of the electrodes was typically 400–600 MΩ when filled with 2 M KAc, 20 mM CG-1 (hexapotassium salt), 10 mM HEPES, 8 mM KCl, 20 mM ATP, 2 mM GTP and 2 mM MgCl2. Neurobiotin was iontophoresed by injecting depolarizing current (0.2–0.5 nA) for 5–20 min at a frequency of 1 Hz with 30% duty cycle from quartz electrodes filled with 2 M KAc and 2% neurobiotin. Preparations were kept at 4°C overnight to allow the diffusion of neurobiotin throughout the neurites. Tissues were fixed, processed using a DAB substrate kit (Ratté and Chase 2000), and sectioned by a vibratome. Current injection and membrane potential recording were carried out with an Axoclamp 2 amplifier (Axon Instrument, Foster City, CA). The electrical signals were amplified, filtered, and digitized (8 bit) by a custom data-acquisition system and stored in one of the first few pixels in the image file to ensure accurate temporal alignment with optical recordings of [Ca2+]i. Post hoc data analysis was carried out using commercial software (Igor, WaveMetrics, Lake Oswego, OR). The amplifier headstage (HS-2A, 0.1 gain) and electrode holder were mounted on an MM33 manipulator (Fine Science Tools, Foster City, CA), which was customized to incorporate a motorizer (860A, Newport, Irvine, CA) in place of the micrometer on the x axis. Cell penetration was achieved by passing a high-frequency oscillating current (1 ms in duration) through the electrode. The current oscillation was caused by a brief overcompensation (“buzzing”) of the pipette capacitance by the negative capacitance circuit in the amplifier.

Calcium dynamics was measured with a custom-built 2PLSM as described previously (Denk and Svoboda 1997). Briefly, sample observation, two-photon laser scanning and fluorescence detection were performed with a water-immersion lens (Achroplan, ×40, 0.75 NA, Zeiss). Beam scanning was controlled by a pair of galvanometer mirrors (Model 6800HP, Cambridge Technology, Cambridge, MA) driven by custom software (R. Stepnowski, Lucent Technologies). The light source was a Ti:sapphire laser (Tsunami; Spectra-Physics) that provided pulses with width <200 fs and repetition rate at 100 MHz. A wavelength of 830 nm was used to excite the calcium green dye. Fluorescence was detected by an intensified photodiode (Intevac). All Ca2+ dynamics data were acquired in line-scan mode with 2-ms time resolution and were analyzed using Igor with custom procedures. Data are presented as averages ± SD unless specified otherwise.

RESULTS

Two types of bursting neurons in the Limax PC lobe

Previous experiments have shown two major classes of neurons in the Limax PC lobe, bursting and nonbursting neurons (Kleinfeld et al. 1994; Watanabe et al. 1998). Nonbursting neurons receive periodic inhibitory inputs and produce spikes that are not phase-locked to the ensemble LFP recording. The bursting neurons show periodic bursts of APs that are phase locked to the LFP (Delaney et al. 1994; Gelperin and Flores 1997; Gelperin et al. 1996; Kleinfeld et al. 1994). In this study, we employed 2PLSM to investigate the temporal dynamics of Ca2+ transients in the bursting neurons. CGD-coated microelectrodes (Gelperin and Flores 1997) were used to label the PC lobe. Bursting neurons have multiple processes extending several hundred micrometers confined to the cell layer while nonbursting neurons have a single neurite extending directly into the adjacent neuropil (Ratte ´ and Chase 2000; Watanabe et al. 1998). Thus any CGD-labeled cell that is found in the cell layer 100 μm from the dye deposition site can be assumed to be a bursting neuron. Indeed all measurements from labeled cells >100 μm from the dye deposition site showed Ca2+ transients phase locked to LFP recordings as is typical of bursting PC neurons (see following text).

Based on their fine morphological properties, we discovered that there are two different types of bursting neurons. One class, denoted as smooth bursting neurons (SBs), has a soma diameter of 14.6 ± 2.3 μm (n = 11; all the soma size measurements refer to the largest diameter unless indicated otherwise) and no varicosities in their processes (Fig. 1A). SBs are not evenly distributed throughout the PC lobe but occur with much higher density near the base than in the apical region (data not shown). The bursting neurons reported in Watanabe et al. (1998) appear to have similar properties in both location and soma size as the SBs in our study. The second class of bursting neurons, which we denote as varicose bursting neurons (VBs), has button-like structures along their neurites and a smaller soma diameter (11.6 ± 0.9 μm, n = 3; Fig. 1B). Figure 1B displays a VB that was identified by sharp-electrode voltage recording and was subsequently filled with neurobiotin by current injection. Typically the somata of VBs were not labeled by CGD, perhaps due to the smaller diameter of their neurites (as small as 0.1 μm, see Fig. 1D) or the existence of a diffusion barrier at the initial segment of the neurite, as found in cultured rat hippocampal neurons (Winckler et al. 1999). The distribution of CGD-labeled varicosities appears to be the same throughout the PC lobe (data not shown). Figure 1C shows the distribution of distances between consecutive varicosities for a sample of VBs with an average distance 15.6 ± 7.5 μm (data pooled from 8 different PC lobes). The distribution histogram has peaks at 8, 16, 24, and 32 μm, which are multiples of 8 μm, the averaged somata diameter of nonbursting neurons (Gelperin et al. 1993). Figure

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1D shows a scanning electron micrograph of a branched neurite of a VB apposed to the somata of nonbursting neurons in the cell layer of the PC lobe. Several varicosities can be seen. The VB shown in Fig. 1B has ~40 varicosities in all branches, suggesting that this cell may contact 40 nonbursting neurons through chemical synapses. However, synapses are not restricted to varicosities as SB neurons lacking varicosities are synaptically coupled to the network of bursting neurons.

Concurrent optical and intracellular recordings in bursting neurons

To establish a temporal correlation between membrane potential changes and calcium dynamics during AP production, we measured $[\text{Ca}^{2+}]_i$, by monitoring the time course of CGD fluorescence and performed simultaneous intracellular recording from a bursting neuron (Fig. 2A). Once a bursting neuron was identified and a stable resting potential was obtained, CG-1 was injected into the soma using hyperpolarizing current. The 2PLSM line scan technique (Helmchen et al. 1999; Svoboda et al. 1997; Yuste and Denk 1995) was used to obtain dynamics of $\text{Ca}^{2+}$ transients with high temporal resolution (2 ms). However, whether the recording is from a VB or SB could not be determined in this case due to either slow diffusion of the CG-1 dye in these cells or an intracellular diffusion barrier at the neurite origin. Up to 3 h after dye injection, no neurites were observed in three dye-injection experiments that were successful as judged by the staining of the soma. The resting potentials were $-64 \pm 9 \text{ mV}$. The decay time constants of the fluorescent transients ranged from 300 to 900 ms with peak amplitudes from 17 to 38%. It should be noted that the onset of the rising phase of $\text{Ca}^{2+}$ transients coincides with the onset of

![FIG. 1. Smooth and varicose bursting neurons. A: a smooth bursting neuron labeled with calcium green dextran. It is a composite picture of multiple images taken using a two-photon microscope. The focus of the microscope was adjusted so that the neurite was always in focus. $[\text{Ca}^{2+}]$ dynamics of this cell was examined and the firing properties indicated that it is a bursting neuron. B: a varicose bursting neuron filled with neurobiotin. Intracellular electrical recording indicated that this is a bursting neuron. Neurobiotin was injected, and standard histological processing was performed to reveal the morphology. The photograph is a composite picture created from 3 different focal planes by using a DIC microscope to take the original pictures and commercial software (Photoshop) to create this camera lucida-like picture. The PC lobe was sectioned along the apical-basal axis at 150 μm with a vibratome. The side view of the section is shown with cell layer on top and neuropil region on the bottom. Apex is to the left. C: distance distribution between consecutive varicosities in the neurites of varicose bursting neurons (VBs). Images of the VBs were obtained by using the two-photon microscope, and distance between consecutive varicosities was measured with NIH image software. The distribution histogram has peaks at 8, 16, 24, and 32 μm. Data are pooled from 8 cells from 8 different PC lobes. D: electron micrograph shows the contact between varicosities and somata of nonbursting neurons. Tissue was freeze-dried and coated with gold for examination in a scanning electron microscope by standard methods. Calibration bar is 20 μm in A and B, 2 μm in D.](http://jn.physiology.org/Archive/jn/1997/086.12/979f1a.jpg)
the first AP in the burst of APs in the intracellular recording. In Fig. 2B, the first AP in each burst was used to align the Ca\(^{2+}\) transients and averaged traces were generated from the aligned [Ca\(^{2+}\)] transients.

We then compared the change of the membrane potential with [Ca\(^{2+}\)]. In Fig. 2C, the differentiated \(dF/F\), as a measure of Ca\(^{2+}\) influx, is plotted together with the membrane potential. Peaks of the first APs are remarkably well aligned with peak of the \(\partial (dF/F)/\partial t\) signal. Thus timing of APs can be deduced with millisecond precision from the derivative of fluorescence measurements, \(\partial (dF/F)/\partial t\). We found that an accurate way to obtain the times of the peaks of the \(\partial (dF/F)/\partial t\) signal is by fitting a sum of multiple Gaussian curves, which is practical and numerically stable (Fig. 2D). Even though the fluorescence signal per se is noisier in neurites than in the soma, the surface-to-volume ratio and consequently the relative changes are much larger in neurites and better timing data can ultimately be obtained in neurites (see Fig. 4). We believe that the APs in bursting neurons are mediated by Ca\(^{2+}\) currents mainly because the duration of the APs, \(~10\) ms, is typical of Ca\(^{2+}\) APs (Baker et al. 1971; Gelduldig and Junge 1968). The presence of voltage-gated Ca channels is evident from the close link between measured cellular depolarization and Ca\(^{2+}\) influx.

**LFP traces can be used to align the APs of bursting neurons**

The extracellular LFP recording is a conveniently obtainable signal, and it has been used for temporal alignment of nonsimultaneous intracellular electrical recordings (Kleinfeld et al. 1994). We therefore established that it can be used for temporal alignment of nonsimultaneous optical and/or intracellular electrical recordings. To this end, we performed simultaneous nystatin-perforated patch recording (Delaney et al. 1994; Kleinfeld et al. 1994) from the soma of a bursting neuron and LFP recording from the PC lobe (Fig. 3A). The intracellular recording revealed a burst of three APs followed by a hyperpolarization phase during each cycle of the oscillation. To explore the precision of relative timing between the APs and the LFP signal, the time point of each negative peak of the LFP was used to align the intracellular traces. This resulted in the APs in successive bursts being well aligned (Fig. 3B). Figure 3C shows the averaged trace of the aligned APs in Fig. 3B. The half-width of the APs in the averaged trace is almost the same as those before averaging.

These results demonstrate that the negative peaks of the LFP can be used to align successive optical (\(dF/F\)) signals to allow accurate timing in optical recordings.
accurate temporal comparison of optical signals from different sites with an accuracy of \( \pm 2 \) ms. The application of such an alignment procedure to \([Ca^{2+}]_i\) recordings is presented in Fig. 4, where the top panel shows \(dF/F\) of the fluorescent signal and the bottom panel shows the LFP traces. The superimposed traces (Fig. 4, B and E) from the different oscillation cycles overlap well when the negative peaks of the LFP are used for alignment of each oscillation cycle. The averaged \(dF/F\) traces display three peaks, which correspond to the three APs per burst recorded in most bursting neurons.

\begin{itemize}
\item \textit{Initiation and propagation of} \(Ca^{2+}\) \textit{APs in bursting neurons}
\end{itemize}

We now used our ability to precisely align nonconcurrent recordings to compare the arrival of AP at different locations in a neuron. Figure 5A shows \(Ca^{2+}\) imaging from five different points along the neurite of a SB aligned by a common LFP recording. The \(dF/F\) traces suggest that the earliest rise occurs at location d and then spreads bilaterally to c-b-a and e, respectively. This suggests an AP initiation zone at or near point d. Similar results were also obtained from VBs. Figure 5B presents the propagation speed of APs in both VBs and SBs. The timing of APs was obtained from the first peak of \(\delta(dF/F)/\delta t\) by fitting with Gaussian curves (see Fig. 2D). To avoid the erroneous estimates that result if an initiation zone was in between two measuring points, measurements from around apparent initiation zones were excluded from Fig. 5B. We found consistently that the conduction velocity was several-fold higher in VBs \((4.3 \pm 0.2 \text{ SE}) \mu m/ms, n = 9\) than in SBs \((1.3 \pm 0.2 \mu m/ms, n = 6\).

A further test of our alignment procedure was provided by the following experiment where we performed simultaneous

\begin{itemize}
\item \textit{measurements on VB neurites using the LFP for temporal
\end{itemize}

registration. The \(dF/F\) measurements from two different varicosities on the same neurite. Figure 6 shows two varicosities, 77 \(\mu m\) apart on the same neurite of a varicosne bursting neuron that displayed a time difference of 18 ms. After the line-scan experiments, multiple images with higher resolution were taken at different focal planes and a three-dimensional reconstruction was performed to confirm that the two varicosities belong to the same neurite (data not shown). The calculated conduction velocity between these two varicosities is 4.3 \(\mu m/ms\), consistent with measurements on VB neurites using the LFP for temporal registration.

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It has been shown previously that odor stimulation of the olfactory epithelium in a nose-brain preparation causes a collapse of wave propagation in the PC lobe and all regions of the PC lobe are excited synchronously (Delaney et al. 1994; Kleinfeld et al. 1994). We therefore investigated the Ca\(^{2+}\) dynamics of bursting neurons in a nose-brain preparation. Figure 7A shows the schematic arrangement. The LFP recording was obtained from the apical region of the PC lobe. CGD-labeled

\begin{itemize}
\item \textit{FIG. 6. Temporal difference between 2 varicosities on the same neurite. The 2 varicosities (a and b) are 77 \(\mu m\) apart on the neurite of a varicosne bursting neuron. Line scan mode of the two-photon microscope was used to image these 2 varicosities. The cross-correlation of \(Ca^{2+}\) dynamics from the 2 points indicates a time difference of 18 ms. A: fluorescent signals (\(dF/F\)) from the varicosities a and b. B: averaged \(Ca^{2+}\) transients from 18 oscillation cycles. The individual and averaged \(dF/F\) signals shown in the bottom have been smoothed with a binomial filter (width = 100). For averaging, the averaged peak times from the 2 varicosities are used to align different oscillation cycles. Inset: picture is inverted in gray scale; black indicates higher fluorescent brightness than white.}
\end{itemize}
bursting neurons in the basal area of a PC lobe were again imaged with the line-scan technique to monitor their Ca$^{2+}$ dynamics. The odor response of a smooth bursting neuron is presented in Fig. 7B: → points to the extra peaks in the basal fluorescence signal that were absent from the apical LFP recording. Similar results were obtained from two other SBs and one VB in separate preparations. When the LFP electrode was moved to the basal region of the PC lobe, it showed similar supernumerary peaks as the d$F$/d$F$ signal.

**Discussion**

The coupled network of bursting neurons in the PC lobe of *Limax* is responsible for the oscillation (0.7 Hz) in the LFP and the activity wave which propagates from apex to base (Delaney et al. 1994; Kleinfeld et al. 1994; Watanabe et al. 1998). The bursting neurons are coupled by glutamatergic chemical synapses and by gap junctions (Wang et al. 2000). The bursters at the apical pole of the PC lobe have a faster burst frequency than the bursters at the basal end, which suggests a possible mechanism for wave propagation (Ermentrout et al. 1998). It remains to be determined, however, if VBs and SBs are equally involved in wave propagation. The wave propagation speed measured optically with a voltage-sensitive dye was 1.1 mm/s (Kleinfeld et al. 1994) as compared with AP conduction velocity in neurites of SBs (1.3 ± 0.2 μm/ms) and VBs (4.3 ± 0.2 μm/ms) obtained in our experiments. Oscillations in LFP and apical-basal wave propagation have been recorded in several other terrestrial slug and snail species (Gelperin et al. 2000; Inoue et al. 1998; Kawahara et al. 1997; Kimura et al. 1998; Nikitin and Balaban 1999). Odor-elicited oscillations were first described in hedgehog olfactory bulb (Adrian 1942) and subsequently in a variety of vertebrate (Adrian 1950; Delaney and Hall 1995; Hughes and Mazurowski 1962; Lam 2000) and invertebrate (Gelperin 1999; Laurent and Naraghi 1994) species. Stimulus-induced wave propagation has been documented in turtle olfactory bulb (Lam et al. 2000) and visual cortex (Precht et al. 2000).

The conduction velocity of APs in the processes of individual bursting neurons would be expected to be faster than the propagation speed of the overall wave. Wave propagation results from the interaction of several processes, synaptic integration as well as neuritic conduction. There are also plausibly conduction pathways from cell layer to neuropil sites and back again into the cell layer via the NB to B cell excitatory synapses that could be essential parts of the wave propagation mechanism. Also, the B cell neurites run at various angles relative to the apical-basal axis, as shown in Fig. 6. If the B cell neurites measured in our experiments were running at an angle to the apical-basal axis and required some chemical synaptic transmission to relay their excitation both along the cell layer and into the neuropil, one could have a plausible explanation for the difference between 1.3 mm/s for the slower B cell neurite propagation speed and the wave speed of 1.1 mm/s. The discrepancy between the faster B cell propagation speed (4.3 mm/s) and the overall wave propagation speed (1.1 mm/s) suggests that the faster B cells are doing something different from just propagating excitation for the wave along the apical-basal axis. For example, they may function to make sure that the wave occupies the full lateral extent of the PC as it moves along, i.e., they serve to propagate excitation in the axis transverse to the apical-basal axis to insure that the wave front occupies the full width of the PC lobe.

Several observations are consistent with the possibility that an influx of Ca$^{2+}$ ions causes the membrane depolarization detected by intracellular recordings. The waveforms of electrically recorded spike activity are very similar to that of the differentiated d$F$/d$F$ signal (Fig. 3). The half-width of the spikes from intracellular recording is ~10 ms, also suggesting that this is a Ca$^{2+}$ spike because the half-width of Na$^{+}$ APs is ~1 ms in squid giant axon. Incubation of the PC lobes with 100 μM TTX for 1 h did not change the LFP oscillation nor the spike pattern of the bursting neurons measured by intracellular recordings. Replacement of extracellular Na$^{+}$ with Tris did not prevent wave propagation in the PC lobes (Wang, Flores and Gelperin, unpublished data). Furthermore depolarization of the membrane by injecting current through the intracellular electrode increased the fluorescence intensity of a CG-1-labeled bursting neuron (Wang and Gelperin, unpublished data), suggesting that there are voltage-activated Ca$^{2+}$ channels on the membrane. Future experiments with specific channel blockers and Ca$^{2+}$ chelators will clarify this issue.

Each bursting neuron in the electrically and chemically coupled network of bursting neurons inhibits a group of nonbursting neurons in the PC lobe, producing inhibitory postsynaptic potentials (IPSPs, 7–9 mV in amplitude) in each nonbursting neuron with each burst (Gelperin 1994; Kleinfeld et al. 1994; Watanabe et al. 1998). The IPSP is thought to be glutamatergic (Watanabe et al. 1998). The inhibitory synaptic currents driven in multiple nonbursting neurons coherently during each presynaptic burst produce the extracellular current flow that is recordable as the LFP (Gelperin et al. 1993). Ultrastructural analysis of varicosities in the *Helix* PC lobe indicates that these varicosities contain specialized structures indicative of presynaptic terminals (Ratte´ and Chase 2000). Two-photon Ca$^{2+}$ imaging of varicosities of neurons in the pyloric network of spiny lobster demonstrates that varicosities are sites of [Ca$^{2+}$]i accumulation (Kloppenburg et al. 2000). The ~40 varicosities seen in all neurite branches of a carefully analyzed VB (Fig. 1B) suggest that this neuron may innervate ≥40 different nonbursting neurons. This number is also consistent with the observation that <5% of the PC neurons are bursting neurons.

The PC lobe has two modes of activity, waves and “blinking,” the latter a mode in which the apical-basal phase gradient is greatly reduced, leading to near synchronous activity throughout the apical-basal extent of the PC lobe (Ermentrout et al. 1998; Kleinfeld et al. 1994). Mode switching appears to occur within one cycle of the LFP oscillation because states intermediate between waves and blinking are not observed (cf. Fig. 5 in Ermentrout et al. 1998). Similarly, bursting cortical networks have been observed to show very short phase delays (~3 ms) over distances of several millimeters (Gray et al. 1989); these may be associated with spike doublet firing by inhibitory interneurons (Traub et al. 1996). When the inhibitory burster neurons in the PC lobe of *Limax* produce double bursts, the LFP recording shows double events and the apical-basal latency is much reduced for the first LFP event of each pair of LFP events (Ermentrout et al. 1998; Kleinfeld et al. 1994).

PC lobes receive direct input from the olfactory receptor neurons (Chase and Tolloczko 1993). Odor stimulation on the
nose epithelium is capable of changing the mode of wave propagation to blinking in the PC lobe. In response to odor stimulation at the nose in a nose-brain preparation, additional peaks were seen in [Ca$^{2+}$], of bursting neuron at the base different from the LFP recording at the apical region (Fig. 7B).

This result suggests that odor stimulation may change the excitability of bursting neurons in the basal PC lobe but not the apical PC lobe. Considering the fact that SBs are mostly situated in the basal region and VBs are evenly distributed, we believe that the observed odor response is mediated by the SBs. It is also possible that a VB in the basal PC lobe, synaptically driven by a SB, shows the supernumerary peaks in response to odor stimulation, which in turn causes supernumerary peaks in LFP at the base because many nonbursting neurons are innervated by VBs.

Networks of inhibitory interneurons in mammalian cortex have been shown to be coupled with both chemical and electrical synapses (Galarreta and Hestrin 1999; Gibson et al. 1999), both of which are believed to be critical to the ability of coupled inhibitory interneurons to promote synchrony in activity of groups of principal cells (Cobb et al. 1995; Michelson and Wong 1994; Strata et al. 1997). Temporal correlations among responding cortical principal neurons resulting from coupled inhibitory neuron activity may be the basis for feature binding and pattern recognition (Gray 1999; Hopfield 1999; Singer 1999).

The VBs have two or more neurites extending over several hundred micrometers contacting several 10s of nonbursting neurons (cf. Fig. 1B) (Ratté and Chase 2000). The conduction of activity in such highly branched small-diameter neuritic networks could be subject to frequency-dependent block at branch points or contain multiple sites of spike initiation as demonstrated in other highly branched interneurons (Antic and Zecevic 1995; Baccus et al. 2000; Gu 1991; Zecevic 1996). Gap junctions between burster neurons must be located on their neurites where the change in [Ca$^{2+}$], occurring during AP production could cause an increase (Baux et al. 1978) or decrease (Pereda et al. 1998) in conductance. The PC lobe also contains symmetrical chemical synapses (McCarragher and Chase 1985) although the identity of the PC cells forming the symmetrical synapses is not known. The use of symmetrical chemical synapses and (nonrectifying) gap junctions to couple the burster neurons could be related to the ability of the burster network to conduct activity both from apex to base and from base to apex, the latter direction of propagation seen after appropriate pharmacological treatments in vitro (Kleinfeld et al. 1994; Wang, Flores, and Gelperin, unpublished results).

The modulation of burster-burster coupling may underlie the large changes in the amplitude of the LFP recorded with fine wire electrodes implanted in the PC lobe in vivo (Cooke and Gelperin 2001).

Electron micrographs of varicose bursting neurons in the Helix PC lobe reveal postsynaptic specializations in neuritic segments between varicosities and symmetrical chemical synapses in varicosities (Ratté and Chase 2000). The network of bursting neurons in the Limax PC lobe (revealed by sulforhodamine labeling) suggests that bursting neurons contact each other through their neurites (Wang, Flores, and Gelperin, unpublished observations). These anatomical results are consistent with our observation that APs are initiated in neurites rather than in somata. Several classes of amacrine (Massey and Mills 1999; Wright and Vaney 2000) and horizontal (He et al. 2000) cells in the vertebrate retina are coupled by electrical and inhibitory chemical synapses (Becker et al. 1998; Vaney 1999). The electrical synapses contribute to the spread of activity from cells directly activated by a visual stimulus while the inhibitory chemical synapses counteract this spread of activation from the site of the direct visual activation (Roska et al. 2000). The coupling strength between bursting neurons through chemical and electrical synapses could be modulated independently by different factors such as olfactory inputs or neuromodulatory inputs, reflecting physiological variables such as the state of satiation or hydration. APs are used to relay excitation from burster neuron to burster neuron presumably because the long thin neurites of burster neurons would not sustain electrotone potentials over distance required for effective burster-burster coupling. Furthermore refractory period of APs would allow wave propagation in only one direction consistent with the hypothesis that phase waves are used by the Limax PC lobe (Ermentrout et al. 1998).

Activity wave propagation like that recorded in the Limax PC lobe has also been observed in turtle visual cortex in response to visual stimuli (Prechtl et al. 1997). The propagating activity in turtle visual cortex, as in Limax PC lobe, is most likely due to local coupling in a network of coupled oscillators. Activity waves arising from stable phase differences in a network of coupled oscillators occur in Limax PC lobe (Ermentrout et al. 1998) and in the swim circuit of the lamprey (Cohen et al. 1992; Deliagina et al. 2000) and of the leech (Brodfuehrer et al. 1995). Activity waves can be produced by other mechanisms, such as local pacemaker regions periodically stimulating excitation along a coupled network, as in developing retina (Butts et al. 1999; Feller et al. 1997; Meister et al. 1991). The Limax PC lobe has some similarity to developing retina as the PC lobe incorporates synaptic connections from new olfactory receptors throughout life (Chase and Rieling 1986) and synaptic connections from new PC neurons born after hatching (Zakharov et al. 1998), as in the vertebrate analogue of the PC lobe, the olfactory bulb (Gelperin 1999).

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