Phase Relationships Between Segmentally Organized Oscillators in the Leech Heartbeat Pattern Generating Network

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Masino, Mark A. and Ronald L. Calabrese. Phase relationships between segmentally organized oscillators in the leech heartbeat pattern generating network. J Neurophysiol 87: 1572–1585, 2002; 10.1152/jn.00336.2001. Motor pattern generating networks that produce segmentally distributed motor outflow are often portrayed as a series of coupled segmental oscillators that produce a regular progression (constant phase differences) in their rhythmic activity. The leech heartbeat central pattern generator is paced by a core timing network, which consists of two coupled segmental oscillators in segmental ganglia 3 and 4. The segmental oscillators comprise paired mutually inhibitory oscillator interneurons and the processes of intersegmental coordinating interneurons. As a first step in understanding the coordination of segmental motor outflow by this pattern generator, we describe the functional synaptic interactions, and activity and phase relationships of the heart interneurons of the timing network, in isolated nerve cord preparations. In the timing network, most (75%) of the coordinating interneuron action potentials were generated at a primary spike initiation site located in ganglion 4 (G4). A secondary spike initiation site in ganglion 3 (G3) became active in the absence of activity at the primary site. Generally, the secondary site was characterized by a reluctance to burst and a lower spike frequency, when compared with the primary site. Oscillator interneurons in G3 inhibited spike activity at both initiation sites, whereas oscillator interneurons in G4 inhibited spike activity only at the primary initiation site. This asymmetry in the control of spike activity in the coordinating interneurons may account for the observation that the phase of the coordinating interneurons is more tightly linked to the G3 than G4 oscillator interneurons. The cycle period of the timing network and the phase difference between the ipsilateral G3 and G4 oscillator interneurons were regular within individual preparations, but varied among preparations. This variation in phase differences observed across preparations implies that modulated intrinsic membrane and synaptic properties, rather than the pattern of synaptic connections, are instrumental in determining phase within the timing network.

INTRODUCTION

Most rhythmic motor patterns are programmed in part by neural circuits called central pattern generators. When they control motor patterns involving segmentally repeated muscles or appendages, e.g., swimming in lamprey (Cohen 1987a,b; Grillner et al. 1991, 1995), leech (Friesen and Pearce 1993), and crayfish (Mulloney et al. 1993; Murchison et al. 1993) and peristaltic heartbeat activity in leeches (Calabrese and Peterson 1983; Peterson 1983a,b), these pattern generators are distributed along several segments of the nerve cord. They have often been characterized as being organized as autonomous segmental oscillators, which are coupled by intersegmental synaptic connections that coordinate the segmental oscillators so that a stable intersegmental pattern is produced. Often there are constant phase differences in motor activity between segments, which lead to a traveling wave of activity along the nerve cord that then produces a traveling wave of muscular contraction along the body axis.

The core timing network of the heartbeat central pattern generator of the leech has been characterized as comprising two coupled segmental oscillators (Calabrese et al. 1995; Peterson 1983a,b). This network has been described in cellular detail (Calabrese et al. 1995), and thus it affords an opportunity for a cellular analysis of the mechanisms underlying coordination between coupled oscillators. We undertake this analysis in three stages. In this first paper, we analyze the functional synaptic interactions between coordinating and oscillator interneurons of the timing network and quantify their activity and phase relationships. We show that the phase relationships within this timing network are flexible so that different preparations express different intersegmental phase relationships. In the second paper (Hill et al. 2002), we provide a plausible model based on the data from this paper that can account for these flexible phase relationships, provides mechanistic insights into how they are generated, and makes specific experimentally testable predictions. In the third paper, some of these predictions are tested under conditions of mutual entrainment (closed loop) (Masino and Calabrese 2002).

METHODS

Animals and solutions

Leeches (Hirudo medicinalis) were obtained from commercial suppliers (Leeches USA, Westbury, NY, and Biopharm, Charleston, NC) and maintained in artificial pond water at 15°C. After the animals were anesthetized in cold saline, ganglia were dissected and pinned (ventral surface up) in small Petri dishes filled with silicone elastomer (Sylgard, Dow Corning, Midland, MI). Ganglia were desheathed using fine scissors. Heart interneurons were first identified based on soma size and soma location in the ganglion and ultimately identified by their characteristic bursting activity (Fig. 1B). The desheathed preparation was superfused continuously with normal leech saline containing (in mM) 115 NaCl, 4 KCl, 1.8 CaCl2, 10 glucose, and 10 HEPES buffer, adjusted to pH 7.4 with NaOH. Depending on the experimental protocol used, preparations consisted of chains of ganglia either from the head brain to the fourth ganglion (HB–G4) or from the third to fourth ganglia (G3–G4).

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Extracellular and intracellular recordings, stimulating and dye injection techniques

For intracellular recordings from heart interneurons, we used sharp intracellular electrodes (20–25 MΩ filled with 4 M KAc, 20 mM KCl) to record the heart interneurons following the methods described in Nadim and Calabrese (1997).

For extracellular recordings from heart interneurons, we used suction electrodes filled with normal saline. Electrodes were pulled on a Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA) from borosilicate glass (1 mm OD, 0.75 mm ID, A. M. Systems) and placed in a suction electrode holder (E series, Warner Instruments, Hamden, CT). To ensure a tight fit between the cell and electrode, the electrode tips had a final inner diameter of ~20 μM, approximately the diameter of heart interneuron somata. The electrode tip was brought in contact with the cell body, and light suction was
applied using a syringe until the entire cell body was inside the electrode. Extracellular signals were monitored with a differential AC amplifier (model 1700, A-M Systems, Carlsborg, WA) at a gain of 1,000 with the low- and high-frequency cutoff set at 100 and 1,000 Hz, respectively. Noise was reduced with a 60-Hz notch filter, and a second amplifier (model 410, Brownlee Precision, Santa Clara, CA) amplified the signal appropriately for digitization.

For dye injection, chains of ganglia were removed and heart interneurons penetrated with sharp intracellular electrodes filled with dextran-conjugated tetramethylrhodamine (Molecular Probes, Eugene, OR; 4% wt/vol in 0.2 M KAc). Hyperpolarizing current pulses (~1–3 nA, 500-ms duty cycle, ~15–60 min) delivered the dye into the cell bodies. The ganglia were incubated overnight in Leibovitz’s L-15 culture media (GIBCO, Grand Island, NY) at 10°C to allow the dye to diffuse from the soma in the injected ganglion into the next posterior ganglion via the intersegmental axon. Ganglia were fixed in paraformaldehyde (4% wt/vol in phosphate-buffered saline, Sigma, St. Louis, MO) and mounted in glycerol (80% vol/vol in 20 mM NaHCO₃). Each ganglion containing dye-filled processes within the preparation was imaged at ×20 (0.5 na) and ×40 (1.3 na) magnification using a confocal microscope (MRC 1024, BioRad). A stacked series of images (1 μM slices) were reconstructed as a single image (Fig. 2). When necessary, multiple images of each ganglion were combined into a montage using imaging software (Photoshop 5.0, Adobe).

Data acquisition and analysis

Data were digitized using a digitizing board (DigiData 1200 Series Interface, Axon Instruments, Foster City, CA) and acquired using pCLAMP software (Axon Instruments) on a personal computer (PC). A spike train analysis program, written in Matlab (Mathworks, Natick, MA), was used to analyze the data on a Sun SPARCStation Ultra-1 and a PC.

In the analysis program, spikes were detected with a discrimination window. When voltage crossed a lower threshold value but did not exceed an upper threshold, a spike event was detected and was indicated by a raster point above the spike (Fig. 8A). The upper threshold eliminated transient artifacts in the recording. To prevent

FIG. 2. Heart interneuron morphology and electrical activity. The top and bottom panels represent 2 different preparations, each iontophoretically injected with a rhodamine-dextran dye (see METHODS). All preparations were imaged ventral side up and are shown anterior upward. Each image was reconstructed from a stacked series of ~1 μM slices. The top panels show the soma and branching pattern of a typical coordinating interneuron [HN(R,2)] in G2 (top left) and its intersegmental axon [HNₓ(R,2)] in G3 (top right). Notice the coordinating interneuron had a single neuritic branch coming off the primary neurite. Inset: typical intracellular recording from the soma of a different coordinating interneuron. Small-amplitude action potentials indicate that spikes were initiated at some point distal to the soma, and the absence of synaptic potentials in the trace indicate this coordinating interneuron did not receive synaptic inputs in G2. The bottom panels show the soma and branching pattern of a typical oscillator interneuron [HN(R,3)] in G3 (bottom left) and its intersegmental axon [HNₓ(R,3)] in G4 (bottom right). The oscillator interneuron branched extensively in its ganglion of origin. Inset: typical intracellular recording from the soma of a different oscillator interneuron. The large-amplitude action potentials indicate that spikes were initiated near the soma, and the presence of synaptic potentials in the trace indicates that G3 oscillator interneurons cells received synaptic contacts in G3. Numerous fine branches coming off the intersegmental axon in more posterior ganglia were observed in both a typical coordinating (top right) and a typical oscillator (bottom right) interneuron.
multiple detection of the same spike, a refractory period (20 ms), during which spikes could not be recognized, was applied after each detected event. To ensure that all spikes were detected, the refractory period was considerably shorter than the shortest interspike interval (~50 ms). Spikes were then grouped into bursts as follows. After an interburst interval (1 s) elapsed without any spikes detected, the next spike event was identified as the first spike of a burst. Subsequent spikes with interspike intervals less than the interburst interval were grouped into that burst. To eliminate the effects of stray spikes in oscillator interneurons, groups of less than 5 spikes were not considered as bursts. In coordinating interneurons, which had fewer spikes per burst than oscillator interneurons, groups of at least 2 spikes were considered bursts. The median spike in each burst was indicated by a symbol above the burst (Figs. 8A and 9, A1 and B1). In this and the subsequent papers in this series, symbols represent heart interneurons from specific ganglia: diamond, G1,2 coordinating interneurons; circle, G3 oscillator interneurons, and asterisk, G4 oscillator interneurons.

The instantaneous spike frequency, defined as the inverse of the interval between consecutive spikes in a burst, of the oscillator and coordinating interneurons was determined and used to calculate the mean spike frequency and the mean maximum spike frequency within each identified burst, which were then averaged across a series of bursts to yield the averaged means (Table 1).

We calculated the observed-to-expected burst ratio of the coordinating interneurons in HB–G4 chain preparations. Because bursts were expected to occur during each silent interval of a G3 oscillator interneuron, the observed-to-expected burst ratio for a given preparation was calculated by dividing the number of bursts observed by the number of bursts expected and expressed as a percentage: [burst ratio = (Number of Observed Bursts/Number of Expected Bursts) × 100].

The analysis program was also used to determine cycle period (T), phase (φ), and duty cycle (D) for each recorded cell (n ≥ 12 consecutive bursts). Cycle period was defined as the interval in seconds from median spike to median spike of consecutive bursts (Fig. 8A), and the mean cycle period (T méd) was determined for each cell (X). The phase of a given heart interneuron was defined on a cycle-by-cycle basis as the time (t) difference between its median spike (t méd) and the median spike of the G4 oscillator interneuron (t méd; phase marker cell). The time difference was then normalized to the cycle period of the phase marker cell and expressed as a percentage: [φ = (t méd − t méd) × 100]. A phase of 100/0% indicated a cell with no phase difference relative to the phase marker cell, while a 50% phase difference indicated an anti-phasic relationship. A positive phase difference indicated a phase lag, while a negative phase difference indicated a phase lead with respect to the phase marker cell. Duty cycle (D) was defined as the percentage of the cycle period occupied by the burst duration (T burst): D = (T burst/T méd) × 100. The mean duty cycle for each interneuron was then displayed as box plots (normalized burst duration) in the phase diagrams (Fig. 10; described below).

Phase diagrams were used to illustrate phase differences between heart interneurons (Fig. 10). The beginning and end of each box plot indicated the average time of the first and last spike, respectively, in a series of bursts relative to the median spike time of the phase marker cell. Error bars indicated the SD around the mean first and last spike in a burst. The average median spike time of the phase marker cell, indicated by a vertical line that bisected the phase box near its midpoint, was positioned at 100/0% phase on the diagram. The mean phase for each heart interneuron was plotted on the phase diagram with respect to the phase marker cell. Error bars indicated the SD around the averaged median spike in a burst. A shift of the average median spike to the right of 100/0% indicated a phase lag, while a shift of the average median spike time to the left of 100/0% indicated a phase lead.

Actograms illustrated the network activity and firing relationships between heart interneurons in the timing network. Actograms were based on raster presentations similar to those used to display circadian activity rhythms (Peterson and Calabrese 1982; Pittendrigh 1974). Each symbol (indexed by ganglion) represented the time of occurrence of the median spike in an interneuron’s burst. The actogram’s reference cycle was defined by the mean cycle period of the phase marker cell in an unmodified ganglion (T méd = coupled period) chain before any experimental manipulation. Time was broken into a series of segments of constant length (reference cycle) that were arranged sequentially, one below the other. When the cycle period of an interneuron was equal to the segment length, then the symbols formed a straight vertical line. When the cycle period was less than the segment length, then the symbols drifted to the left; when it was greater, the symbols drifted to the right. For visual purposes, a duplicate copy of each segment was displayed to the right and shifted up one row in the graph. One-to-one matching of symbols, which were displaced from one another by a regular horizontal interval, indicated a stable phase relationship between interneurons.

RESULTS

The heartbeat pattern generator consists of paired inhibitory heart interneurons in the first through the seventh segmental ganglia (G1–G7). A subset of these heart interneurons, located in G1 through G4, forms the heartbeat timing network (Fig. 1A). Two foci of oscillation in this network have been identified in G3 and G4, where the oscillation is dominated by the reciprocal interactions of the third and fourth pair of heart interneurons, respectively (Peterson 1983a). Reciprocally inhibitory synapses between the bilateral pairs of heart interneurons in these ganglia, combined with an ability of these interneurons to escape from inhibition, pace the oscillation (Angstadt and Calabrese 1989; Peterson 1983a). Thus the heart interneurons in G3 and G4 are called oscillator interneurons. The rhythmic activity of oscillator interneurons consists of a burst of high-frequency firing interrupted by a nearly equal interval of strong synaptic inhibition (Fig. 1B; synaptic potentials are not present in these extracellular recordings). The heart interneurons of G1 and G2 (G1,2) act as coordinating fibers and link the oscillator interneurons in G3 and G4, thus forming the heartbeat network for the system (Peterson 1983b). The coordinating interneurons inhibit the ipsilateral oscillator interneurons and in turn are inhibited by the ipsilat-

<table>
<thead>
<tr>
<th>Heart Interneuron</th>
<th>Mean Spike Frequency, Hz</th>
<th>Mean Maximum Spike Frequency, Hz</th>
<th>Mean Number of Spikes Per Burst</th>
<th>Mean Duty Cycle, %</th>
</tr>
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<tbody>
<tr>
<td>G1,2</td>
<td>4.5 ± 1.4 (35)</td>
<td>6.9 ± 3.1 (35)</td>
<td>9.6 ± 3.2 (35)</td>
<td>27.6 ± 9.1 (35)</td>
</tr>
<tr>
<td>G3</td>
<td>13.4 ± 2.1 (35)</td>
<td>17.9 ± 2.7 (35)</td>
<td>62.4 ± 9.5 (35)</td>
<td>57.1 ± 2.7 (35)</td>
</tr>
<tr>
<td>G4</td>
<td>13.2 ± 1.9 (28)</td>
<td>17.9 ± 2.5 (28)</td>
<td>59.9 ± 7.9 (28)</td>
<td>56.6 ± 3.2 (27)</td>
</tr>
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Values are means ± SD with number of interneurons in parentheses.
eral oscillator interneurons. As a result of the synaptic connections in this network, ipsilateral oscillator heart interneurons in G3 and G4 fire nearly in-phase, while the ipsilateral coordinating heart interneurons fire bursts of action potentials during the interval of inactivity of the G3 and G4 oscillator interneurons (Fig. 1C).

The rhythmic activity of individual coordinating interneurons is similar to oscillator interneuron activity where bursts are followed by an interval of inactivity. However, the bursts in the coordinating interneurons are weaker, characterized by fewer spikes per burst and a clear deceleration in spike frequency. Additionally, while the oscillator interneuron duty cycle is typically ~50% (Hill et al. 2001), the coordinating interneuron duty cycle is typically shorter and varies considerably. The oscillator interneurons in G3 and G4 continue to oscillate normally in isolated, single ganglion preparations. Thus each of these two reciprocally inhibitory heart interneuron pairs, along with the coordinating interneuristor fibers in each ganglion, is considered an autonomous segmental oscillator (Hill et al. 2001; Peterson 1983a).

The coordinating interneurons can initiate spikes at sites located in G4 and G3, respectively (Peterson 1983a). Because there is an asymmetry in the synaptic connections between the G3 and G4 oscillator interneurons onto the coordinating interneurons (Peterson 1983a,b), the timing network can potentially function in two modes, depending on where the coordinating interneurons initiate their spikes (Fig. 1D). The network functions in a symmetric mode if spikes originate at the initiation site in G4 because both G3 and G4 oscillator interneurons inhibit this initiation site (Fig. 1D1). However, if spikes are initiated at the site in G3, the network functions in an asymmetric mode (Fig. 1D2) since only the oscillator interneurons in G3 inhibit this site.

Coordinating heart interneuron morphology and electrical activity

We further characterized the morphology and activity of the coordinating interneurons and their synaptic relations with the oscillator interneurons to determine more precisely the mode in which the timing network functions. The heart interneurons in the timing network (G1–G4) send a single primary neurite from the soma that initially courses anteriorly, then loops back and projects out the ipsilateral posterior connective (Shafer and Calabrese 1981) (Fig. 2, left panels). Intracellular recordings of postsynaptic potentials in heart motor neurons indicate that the oscillator interneurons project at least as far as the 18th segmental ganglion, but the extent of the axon projection of the coordinating interneurons is unknown (Thompson and Stent 1976a,b). The branching pattern of the primary neurite differs between the coordinating and oscillator interneurons. While the oscillator interneurons branch extensively with ~14 primary branches (Fig. 2, bottom left panel), the coordinating interneurons typically have a single primary branch coming off the primary neurite (Fig. 2, top left panel). Numerous fine branches of the intersegmental axons are observed in more posterior ganglia for both types of heart interneurons; note that none of these branches appear to cross midline (Fig. 2, top right and bottom right panels).

The electrical activity of the oscillator and coordinating heart interneurons also differs. In the oscillator interneurons, large-amplitude action potentials and the presence of synaptic potentials in intracellular recordings indicate that they initiate spikes near the soma and receive synaptic inputs in their ganglion of origin (Fig. 2, inset in bottom left panel). In the coordinating interneurons, in contrast, small-amplitude action potentials and the absence of synaptic potentials in intracellular recordings indicate that actively propagated spikes do not invade the regions of the primary neurite near the soma and that they do not receive synaptic inputs in their ganglion of origin (Fig. 2, inset in top left panel).

Both the oscillator and coordinating interneurons are rhythmically active, but their spiking and bursting characteristics differ considerably (Table 1). G3 and G4 oscillator interneurons were very similar in their spiking activities. The duty cycles of the oscillator interneurons were highly regular both within and across preparations. The mean duty cycles of the G3 (n = 35) and G4 (n = 27) oscillator interneurons were similar and varied little across preparations, being 57.1 ± 2.7% and 56.6 ± 3.2% (mean ± SD), respectively. Duty cycles greater than 50% indicated some overlap in activity between bilaterally paired oscillator interneurons (Fig. 1B). G3 and G4 oscillator interneurons show similar spike frequency profiles during their bursts (Fig. 8C). Bursts were characterized by a low (~5 Hz) initial spike frequency, followed abruptly by a period of high (~20 Hz) frequency spiking that slowly decelerated, and ended with a low (~7 Hz) final spike frequency. Both the increase in spike frequency at the start of the bursts and the decrease in spike frequency at the end of the bursts occurred precipitously. The mean spike frequency measured across many preparations was ~13 Hz (Table 1). G3 and G4 oscillator interneurons generated ~60 spikes per burst (Table 1). The number of spikes per burst was related to the burst duration and hence the cycle period, since duty cycle was relatively invariant.

We considered the G1 and G2 (G1.2) coordinating heart interneurons to be equivalent since their spiking activities were similar (Table 1). Given this similarity, we focused on the G2 coordinating interneurons because G2 was easier to desheath than was G1. Nevertheless, the activity of a few (n = 3) G1 interneurons was also recorded. Similar to the oscillator interneurons, the duty cycles of the coordinating interneurons were relatively regular within individual preparations. Unlike the oscillator interneurons, however, the coordinating interneuron duty cycles varied considerably among different preparations and ranged between 12 and 52% with a mean value of 27.6 ± 9.1% (Table 1). The instantaneous spike frequency of the coordinating interneurons was characterized by an initial moderate (~6 Hz) spike frequency that markedly decelerated as the burst proceeded (Fig. 8C). The mean spike frequency measured across many preparations was ~4.5 Hz (Table 1). In addition, the number of spikes per burst was lower in the coordinating interneurons (~10) than in the oscillator interneurons (~60). In the following analysis, all references to multiple heart interneurons refer to ipsilateral interneurons unless stated otherwise.

Previous analysis of the heartbeat network suggested that the primary spike initiation sites for the coordinating interneurons were located in G4, implying that the timing network operated in a symmetric mode (Peterson 1983b). To determine more precisely where spikes in the coordinating interneurons were initiated, we measured the latency between spikes recorded extracellularly from coordinating interneurons and matched inhibitory postsynaptic potentials (IPSPs) recorded intracellularly from G4 oscillator
interneurons. In principle, the coordinating interneurons could initiate their spikes at a site 1) located in G4, 2) located in G3, or 3) located near the soma in the ganglion of origin (G2; Fig. 3). Spikes originating in G4 would propagate anteriorly along the coordinating interneuron axon into and through G3 toward the soma in G2. Such a spike would thus produce an IPSP recorded in the oscillator interneuron in G4 that precedes both the IPSP in the recorded G3 oscillator interneuron and the spike recorded in the coordinating interneuron soma in G2 (Fig. 3, left: G4 Initiation). A spike initiated in the coordinating interneuron in G3, however, would produce nearly simultaneous recordings of the IPSP in the G4 oscillator interneuron and the spike in the soma of the G2 coordinating interneuron since these two sites are equidistant from the site of spike initiation in G3 (Fig. 3, middle: G3 Initiation). Finally, a spike initiated in the ganglion of origin (e.g., G2) would be followed first by an IPSP recorded in the G3 oscillator interneuron and then, with some delay, by an IPSP in the G4 oscillator interneuron (Fig. 3, right: G2 Initiation).

To distinguish among these possibilities, we matched spikes in the G1,2 coordinating interneurons with their IPSPs recorded in G4 oscillator interneurons (Fig. 4). Individual sweeps of the G4 oscillator interneuron’s membrane potential were triggered by spikes in the ipsilateral G1,2 coordinating interneuron and displayed in raster form (Fig. 4B). In this example, 10 of the 13 G2 coordinating interneuron [HN(L,2)] spikes were matched with IPSPs in the G4 oscillator interneuron [HN(L,4)] with an approximately −33 ms latency, which indicated that most spikes were initiated in G4. The IPSP in the 13th sweep followed the G2 coordinating interneuron spike with a latency of −10 ms, which indicated that this spike was initiated in G3. Where the remaining spikes (numbers 1 and 2) were initiated could not be determined. Superimposition (Fig. 4C) and a spike-triggered average (Fig. 4D) of the sweeps from five consecutive coordinating interneuron bursts revealed that the IPSPs grouped with a latency close to −33 ms relative to spikes events in the G2 coordinating interneuron. In this example, 42 of the 59 (~71%) spikes from 5 consecutive bursts (3 of which are shown) were initiated at the site located in G4, whereas 10 (~17%) spikes were initiated at the G3 site. The site of initiation for 7 (~12%) spikes could not be determined. We obtained similar results in all preparations (n = 6) examined, indicating that the primary spike initiation of the coordinating interneurons was located in G4.

Approximately 75% of the spikes across all preparations were unambiguously assigned to the G4 site, whereas only ~6% of the spikes were assigned to the G3 site (the initiation site could not be determined for ~17% of the spikes). In the example shown (Fig. 4A), spikes indicated by an asterisk in each burst were initiated at the secondary site in G3. These results indicate that the coordinating interneuron site of initiation can shift from the primary site in G4 to the secondary site in G3, but did so rarely and usually near the end of the burst when the G4 oscillator interneuron burst overlapped with the coordinating interneuron burst.

Previous analysis (Peterson 1983a,b) indicated that the G3 oscillator interneurons inhibit both spike initiation sites in the coordinating interneurons, whereas the G4 interneurons (owing to the fact that they do not project anteriorly to G3) inhibit only the G4 initiation site of the coordinating interneurons. Our above analysis suggests that spike initiation in the coordinating interneurons might shift if a G4 oscillator interneuron’s burst, but not if a G3 interneuron’s burst, is inappropriately activated during a coordinating interneuron burst. We tested this hypothesis directly for the G4 oscillator interneuron using current pulses to produce the inappropriately timed bursts (approximately +0.5 nA, 1–5 s; Fig. 5). As expected, those spikes initiated in the coordinating interneuron prior to the current pulse originated in G4 (Fig. 5C, Spikes 1 and 2). During the current pulse the spike initiation site shifted; the G4 spike initiation site was turned off, and the G3 site became active.
Once the current pulse was turned off, the coordinating interneuron spike initiation site shifted back to G4. Similar results were obtained in five preparations. These results indicate that the coordinating interneurons are able to "escape" the inhibition of a G4 oscillator interneuron by shifting their spike initiation to their G3 initiation sites, if those sites remain uninhibited by the G3 oscillator interneuron. In contrast, such inappropriately timed bursts (evoked by similar current pulses) in G3 oscillator interneurons silenced the coordinating interneurons completely (Fig. 6; n = 3). These results indicate that the G3 oscillator interneuron inhibits both sites of spike initiation (in G3 and G4) in the coordinating interneurons.

Because our results suggested that the G4 sites of the coordinating interneurons was somehow dominant over the G3 sites, we sought to determine whether there were differences in

**Fig. 4.** Determining that the primary spike initiation site of the G2 coordinating interneuron is in G4. A: simultaneous extracellular (top and bottom traces) and intracellular (middle trace) recordings of ipsilateral oscillator (HN(L,3) and HN(L,4)) and coordinating (HN(L,2)) interneurons. B: raster display of HN(L,4) oscillator interneuron membrane potential traces. Individual sweeps (1 through 13) were triggered by spikes in the HN(L,2) coordinating interneuron [3rd burst of HN(L,2) in A]. The sweeps were 120 ms in duration. Each coordinating interneuron spike occurred at 0 on the time axis. The vertical dashed lines indicate the expected latencies of IPSPs in the oscillator interneuron caused by spikes in the coordinating interneuron. Negative latencies of approximately −33 ms indicate coordinating interneuron spikes were initiated in G4 (see Fig. 3, left), while positive latencies of −10 ms indicate that spikes were initiated in G3 (see Fig. 3, middle). The majority (10 of 13) of the G2 coordinating interneuron spike events were matched with IPSPs in the G4 oscillator interneuron with an approximately −33-ms latency, which indicates that the primary spike initiation site was in G4. The IPSP in the 13th sweep followed the G2 coordinating interneuron spike event with a latency of −10 ms, which indicates that the spike was initiated in G3. In A, asterisks mark spikes generated at the secondary spike initiation site in G3. Spikes generated at an undetermined location are indicated by question marks (?, spikes 1 and 2), while spikes generated at the primary initiation site in G4 are unmarked. C: superimposed sweeps from 5 consecutive bursts, 3 of which are shown in A, of the HN(L,2) (top trace) and HN(L,4) (bottom trace) interneurons. IPSPs in HN(L,4) group at a latency close to −33 ms. D: spike-triggered average of the sweeps in C. Averaged traces yield a prominent trough with a mean latency of nearly −33 ms relative to spike events in the G2 coordinating interneuron. A small kink is observed in the averaged trace at a latency (~6 ms) near the expected latency for IPSPs caused by spikes arising at the G3 site. Asterisks located above dotted line in B–D indicate the expected latency of IPSPs in the G4 oscillator interneuron when spikes are initiated at the secondary site in G3.

(Fig. 5C, Spike 5). Once the current pulse was turned off, the coordinating interneuron spike initiation site shifted back to G4. Similar results were obtained in five preparations. These results indicate that the coordinating interneurons are able to "escape" the inhibition of a G4 oscillator interneuron by shifting their spike initiation to their G3 initiation sites, if those sites remain uninhibited by the G3 oscillator interneuron. In contrast, such inappropriately timed bursts (evoked by similar current pulses) in G3 oscillator interneurons silenced the coordinating interneurons completely (Fig. 6; n = 3). These results indicate that the G3 oscillator interneuron inhibits both sites of spike initiation (in G3 and G4) in the coordinating interneurons.

Because our results suggested that the G4 sites of the coordinating interneurons was somehow dominant over the G3 sites, we sought to determine whether there were differences in
their firing properties that might account for this dominance. We compared spike frequency and the number of spikes per burst at the two sites for nine different preparations (Table 2). We recorded spike activity in G1 and G2 coordinating interneurons from HB–G4 chains. Since the spike initiation at the G4 site accounts for the majority (∼75%) of coordinating interneuron spikes in such preparations, then recording in such a chain can be used to assess the firing characteristics of the G4 neurons.

**FIG. 5.** The effect of activity in G4 oscillator interneurons on spike initiation in G2 coordinating interneurons. A: current pulses (approximately +0.5 nA, approximately 5 s) were used to induce high-frequency firing in a G4 oscillator interneuron during spontaneous bursting in an ipsilateral G2 coordinating interneuron (CM, current monitor). This activity reduced the spike frequency and the number of spikes in the G2 coordinating interneuron. B: time expansion of the selected region in A. C: spikes from the G2 coordinating interneuron were matched to IPSPs from the G4 oscillator interneuron. Spike 1: prior to the current pulse, the latency between the IPSP and the spike was approximately ∼30 ms. Spike 2: the latency remained at approximately ∼30 ms early in the current pulse (∼200 ms). Spike 5: the latency shifted to approximately +5 ms late in the current pulse, which indicates the G2 oscillator interneuron shifted its spike initiation site to G3.

**FIG. 6.** The effect of activity in G3 oscillator interneurons on spike initiation in G2 coordinating interneurons. A: current pulses (approximately +0.5 nA, approximately 5 s) were used to induce high-frequency firing in a G3 oscillator interneuron during spontaneous bursting in an ipsilateral G2 coordinating interneuron. This activity greatly reduced the number of spikes in the burst of the G2 coordinating interneuron (CM, current monitor). B: time expansion of the selected region in A. Like in the case of the corresponding experiment with a G4 oscillator interneuron (Fig. 5), early during the current pulse (∼200 ms) the coordinating interneuron continued to fire, but in contrast, later during the pulse firing ceased.
sucrose knife technique (METHODS in Masino and Calabrese, however, it is necessary to remove G4 from the circuit. To assess the activity at the secondary initiation site in G3, once G4 was removed, the G3 (secondary) spike initiation site of the coordinating interneurons became active in all preparations. A stream of sucrose solution applied to the G3 to G4 junction (1580 M) liberated spike activity at the secondary initiation site in G3 to generate a spike frequency higher than at the primary initiation site (Table 2). In two preparations (Table 2, preparations numbers 2, 5, 7, and 8) had few single spike bursts, whereas preparations with a high ratio (Table 2, preparations numbers 3, 4, and 8) had many single spike bursts, indicating that few bursts were absent (Fig. 7B). Those preparations with low ratios (Table 2, preparations numbers 2, 5, 7, and 8) had few single spike bursts. In seven of nine preparations, the mean spike frequency and the mean number of bursts per burst were significantly lower at the secondary initiation site than at the primary initiation site (Table 2). In two preparations (Table 2, preparations numbers 3 and 8), the secondary initiation site had a higher mean spike frequency than the primary initiation site. Preparation 3 generated fewer spikes/bursts than preparations with low ratios (Table 2, preparations numbers 2, 5, 7, and 8). The observed-to-expected burst ratios of the coordinating interneurons varied among preparations. A low ratio (<50%) reflected the absence of numerous bursts (Fig. 7A), while a high ratio (>80%) indicated that few bursts were absent (Fig. 7B). Those preparations with low ratios (Table 2, preparations numbers 1 and 4) had many single spike “bursts,” whereas preparations with a high ratio (Table 2, preparations numbers 2, 3, 5, 7, and 8) had few single spike bursts. In seven of nine preparations, the mean spike frequency and the mean number of bursts per burst were significantly lower at the secondary initiation site than at the primary initiation site (Table 2). In two preparations (Table 2, preparations numbers 3 and 8), the secondary initiation site had a higher mean spike frequency than the primary initiation site. Preparation 3 generated fewer spikes/bursts than preparations with low ratios (Table 2, preparations numbers 2, 5, 7, and 8). The observed-to-expected burst ratios of the coordinating interneurons varied among preparations. A low ratio (<50%) reflected the absence of numerous bursts (Fig. 7A), while a high ratio (>80%) indicated that few bursts were absent (Fig. 7B). Those preparations with low ratios (Table 2, preparations numbers 1 and 4) had many single spike “bursts,” whereas preparations with a high ratio (Table 2, preparations numbers 2, 3, 5, 7, and 8) had few single spike bursts. In seven of nine preparations, the mean spike frequency and the mean number of bursts per burst were significantly lower at the secondary initiation site than at the primary initiation site (Table 2). In two preparations (Table 2, preparations numbers 3 and 8), the secondary initiation site had a higher mean spike frequency than the primary initiation site. Preparation 3 generated fewer spikes/bursts than preparations with low ratios (Table 2, preparations numbers 2, 5, 7, and 8).

### TABLE 2. Comparison of spike characteristics at the primary (1°) and secondary (2°) spike initiation sites of the G1,2 coordinating interneurons

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Initiation Site of Bursts</th>
<th>Observed Number of Bursts</th>
<th>Expected Number of Bursts (Obs/Exp), %</th>
<th>Single Spike Bursts</th>
<th>Mean Number of Spikes per Observed Burst</th>
<th>Mean Spike Frequency, Hz</th>
<th>Mean Max Spike Frequency, Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>100</td>
<td>6.3 ± 1.1</td>
<td>6.3 ± 1.1</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>16</td>
<td>16</td>
<td>100</td>
<td>2.3 ± 0.6*</td>
<td>0.7 ± 0.5*</td>
<td>2.7 ± 0.5*</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>7.6 ± 0.6</td>
<td>7.6 ± 0.6</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>15</td>
<td>15</td>
<td>100</td>
<td>5.6 ± 0.9*</td>
<td>5.6 ± 0.9*</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>6.9 ± 1.7</td>
<td>6.9 ± 1.7</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>19</td>
<td>19</td>
<td>100</td>
<td>9.3 ± 0.6</td>
<td>9.3 ± 0.6</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>14</td>
<td>14</td>
<td>100</td>
<td>4.1 ± 0.5</td>
<td>4.1 ± 0.5</td>
<td>2.3 ± 1.1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>24</td>
<td>24</td>
<td>100</td>
<td>4.0 ± 1.4</td>
<td>4.0 ± 1.4</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>29</td>
<td>30</td>
<td>96.7</td>
<td>5.5 ± 2.0†</td>
<td>5.3 ± 1.9†</td>
<td>4.3 ± 1.2*</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>17</td>
<td>17</td>
<td>100</td>
<td>8.1 ± 0.9*</td>
<td>8.1 ± 0.9*</td>
<td>4.0 ± 0.6*</td>
</tr>
</tbody>
</table>

Values are means ± SD. Within group comparisons were made using the paired t-test for the number of spikes per burst and spike frequency data sets. Bold text and symbols indicate significant difference in spike activity: *P < 0.001; †P < 0.02; ‡P < 0.007.
spikes per burst at its secondary initiation site, whereas preparation 8 generated a greater number of spikes per burst at its secondary initiation site when compared with their primary initiation site; both of these preparations had high observed-to-expected burst ratios (see following text).

Because there is an asymmetry in the connections between the G3 and G4 oscillator interneurons onto the coordinating interneurons, the timing network potentially functions in two modes, symmetric or asymmetric, depending on where the coordinating interneurons initiate their spikes. Since most of the coordinating interneuron spikes were generated at the G4 (primary) spike initiation site and because both the G3 and G4 oscillator interneurons inhibit this site (Fig. 1A), we conclude that the network functions primarily in the symmetric mode.

Cycle period and phase relationships within the heartbeat timing network

We used simultaneous extracellular recordings from ipsilateral heart interneurons to analyze the activity pattern of the heartbeat timing network. Both cycle period and the phase relationships among interneurons were regular within individual preparations, but differed considerably among preparations (Figs. 8 and 9). A representative preparation with both a regular cycle period (8.1 ± 0.2 s) and a regular G3 to G4 phase difference (16.6 ± 1.6%) is shown in Fig. 8. Similar to results from Arbas and Calabrese (1984) obtained with intracellular recordings from heart motor neurons, the cycle periods of individual preparations when recorded extracellularly were regular over long periods (~1 h). Cycle periods among preparations, however, varied considerably (Figs. 8, 9, and 11B) and ranged between ~6 and 21 s. Similarly, the G3 to G4 phase difference was regular within individual preparations, but varied considerably across preparations (Fig. 11A).

Three examples of the phase relationships observed are shown in the phase diagrams of Fig. 10. In most cases (90 of 131, 69%), the G4 oscillator led in phase (Fig. 10A), and in others (n = 22, 17%) the G3 oscillator led in phase (Fig. 10B), and in others (n = 19, 14%) there was zero phase difference between the segmental oscillators (Fig. 10C). Cycle period and G3 to G4 phase were not correlated (r = 0.03, P = 0.77, n = 131; Fig. 11B).

The phase relation of the G1 and G2 (G1,2) coordinating...
interneurons to the oscillator interneurons was approximately anti-phasic (Fig. 1C). If G1,2 phase was to vary equally with both the G3 and the G4 oscillator interneurons, then in plots of G1,2 phase versus G3 to G4 phase the absolute values of the slopes of the regression lines should be similar and approximately equal to 0.5. When analyzed, however, the slopes of the regression lines differed. The phase relation of the G1,2 coordinating interneurons to the G3 oscillator interneurons was relatively constant ($m = 0.3$; shallow slope of the dotted regression line) when compared with the G4 oscillator interneurons ($m = 0.7$; steep slope of the solid regression line; Fig. 1C). The asymmetry of the connections in the timing network (Fig. 1A), where the G3 oscillator interneurons inhibit both initiation sites of the coordinating interneuron, while the G4 oscillator interneurons inhibit only the G4 site, may account for the relative constancy of G1,2 to G3 phase relationships versus the G1,2 to G4 phase relationships.

Our analysis thus far shows that the G2 coordinating interneurons were most active during the time the ipsilateral G3 and G4 oscillator interneurons were silent, but that there was some overlap in activity between the oscillator and coordinating interneurons (Figs. 1C, 8, A and C, 9, and 10). To assess the extent to which the bursts of the coordinating interneurons filled only the interval in which both ipsilateral G3 and G4 oscillator interneurons were silent, we plotted the G1,2 coordinating interneuron duty cycle against the G3 to G4 phase difference (Fig. 11D). If we assume that the coordinating interneurons are active only whenever both ipsilateral G3 and G4 oscillator interneurons are silent, then the maximum coordinating interneuron duty cycle should occur when the G3 and G4 oscillator interneurons are in-phase (Fig. 10C). Since the mean duty cycle is $-57\%$ for both oscillator interneurons (Table 1), then the maximum coordinating interneuron duty cycle should be $-43\%$. This duty cycle should then decrease linearly as the absolute value of the G3 to G4 phase increases, because the window for coordinating interneuron firing closes as the disparity between G3 and G4 oscillator interneuron increases (Fig. 10A and B). Thus a plot (Fig. 11D; solid line) of the expected G1,2 duty cycle versus G3 to G4 phase difference should form an inverted V with its apex representing the maximum duty cycle ($-43\%$) predicted for the coordinating interneuron when there is no G3 to G4 phase difference. When the actual data are plotted on the same axes, the coordinating interneuron duty cycle was generally shorter than predicted (most points fell below the solid line in Fig. 11D). In general the coordinating interneurons do not appear to fill the window open for their firing.

The analysis of G1,2 coordinating interneuron duty cycle suggests that there is little opportunity for overlap between coordinating interneuron firing and oscillator interneuron firing. This is not always the case as seen in Figs. 1C, 9A, and 10, A and B, where the firing of the G4 oscillator interneuron overlaps with the firing of the G2 coordinating interneuron. Such overlap occurs when both cells are firing at low frequency (Fig. 10, A and B), but significantly even this level of overlap is rarely seen between the firing of the coordinating interneurons and the G3 oscillator interneuron. This difference between the G3 and G4 oscillator interneurons in their overlap with the
coordinating interneurons may account for the phase asymmetry seen in Fig. 11C and in turn may result because the G3 oscillator interneuron inhibits both initiation sites of the coordinating interneurons, while the G4 oscillator interneuron inhibits only the G4 site.

**DISCUSSION**

The leech heartbeat timing network, which consists of two coupled segmental oscillators, provides the oscillatory drive for the heartbeat central pattern generator. Here we have provided a detailed description of the activity patterns and functional synaptic interactions within this network and presented a quantitative analysis of the interactions between oscillator interneurons and coordinating interneurons. These studies provide the background necessary for a mechanistic understanding of phase relation within the network that is pursued in the subsequent two papers.

**Coordinating interneurons can initiate spikes at multiple sites**

The coordinating interneurons of the heartbeat timing network differ considerably in their spiking and bursting properties from the oscillator interneurons with which they interact (Fig. 1C, Table 1). These coordinating interneurons, like neurons in other pattern generating networks that communicate between ganglionic subnetworks, initiate spikes at multiple sites (mollusks, Perrins and Weiss 1998; crustaceans, Nusbaum et al. 1992; annelids, Calabrese 1980; Thompson and Stent 1976a,b). In the coordinating heart interneurons, spikes are generated mostly at a primary initiation site (G4), but a weaker secondary initiation site is revealed in G3 under certain conditions. For example, the secondary site in G3 becomes active after removal of the fourth ganglion from an HB–G4 chain. The observation that, in the G1,2 coordinating interneurons, the primary (G4) spike initiation site dominates the secondary (G3) initiation site suggests differences in the firing properties between these initiation sites (Table 2). The primary initiation site seems more robust than the secondary site since individual spikes as well as bursts of spikes are generated more reliably at the G4 site.

It is unclear, at this point, whether the two initiation sites in the coordinating interneurons play a major role shaping the motor output of the leech heartbeat pattern generator in the intact animal. Nevertheless, these two sites and the asymmetry in the connections from the G3 and G4 oscillator interneurons onto the coordinating interneurons permits the timing network to function in two modes (Fig. 1D). The network functions in the asymmetric mode when spikes are initiated at the primary (G4) site, which is inhibited by both the G3 and G4 oscillator interneurons (Fig. 1D). Conversely, the network functions in the symmetric mode when spikes are initiated at the secondary (G3) site, which is only inhibited by the G3 oscillator interneurons (Fig. 1D). However, since most spikes originate at the primary (G4) initiation site, the system normally functions in the symmetric mode. There are indications, however, that the structural asymmetries in the timing network yield functional asymmetries in its output. For example, the phase of the coordinating interneurons is more tightly held to the G3 oscillator than the G4 oscillator (Fig. 11C). This asymmetry may reflect that the dominance of the G4 site in the coordinating interneurons is incomplete.

Another manifestation of this same asymmetry and lack of complete dominance by the G4 site is less overlap of the G1,2 coordinating interneurons with the G3 oscillator interneurons than with the G4 oscillator interneurons. If spike initiation in the coordinating interneurons shifts from the primary site to the secondary site when the G4 oscillator interneuron is active but the G3 oscillator interneuron is not, then coordinating interneuron firing can overlap with G4 oscillator interneuron activity. Such a situation occurs as the G4 oscillator interneuron’s burst starts when the G4 oscillator interneuron phase leads the G3 oscillator interneuron (Figs. 8, A and C, and 10A). Such spike initiation shifts from the primary to the secondary site were observed when the ipsilateral G4 oscillator interneuron was forced to fire early in the G1,2 coordinating interneuron burst (Fig. 5). In contrast, no shifting occurs when the ipsilateral G3 oscillator interneuron is forced to fire early in the coordinating interneuron burst, because both initiation sites (primary and secondary) are silenced (Fig. 6).

**Phase constancy and lack thereof**

In many segmentally organized pattern generating networks, the phase relationship between segmental oscillators is constant and remains so even as the network cycle period changes (lamprey swimming, Cohen 1987a; Grillon et al. 1993; Sigvardt 1993; leech swimming, Friesen and Pearce 1993; crayfish swimmeret, Mulloney et al. 1993). Phase constant systems ensure the expression of a single wave of activity along the...
body axis that is maintained by the production of proper phase delays between segments. For example, both the leech and lamprey swim networks produce invariant intersegmental phase relationships in the activity between adjacent segments along the length of the nerve cord regardless of the oscillator frequency (lamprey, Cohen 1987a; Grillner et al. 1993; Sigvardt 1993; leech, Friesen and Pearce 1993). In contrast, the intersegmental phase relationship between the coupled segmental oscillators in the isolated leech heartbeat timing network is remarkably flexible and is not correlated with the cycle period of the network oscillation (Fig. 11B). As shown in this study, a G3 to G4 phase difference between segmental oscillators in isolated nerve cord preparations is observed, and these phase differences are regular within individual preparations, but they vary considerably among different preparations. This variation in the phase differences among different preparations (Fig. 11A) implies that the phase relationships in the timing network are established not by the pattern of synaptic connec-

FIG. 11. Analysis of the normal activity and timing relationships among the heart interneurons. A: frequency histogram showing the variability of the G3 to G4 phase relationships across preparations. The G4 oscillator interneurons led in phase ($\phi_3 - \phi_4 > 0$) in ~70% of the preparations; the median phase difference was 6.1%. B: cycle period and the G3 to G4 phase difference were not correlated. The G3 to G4 phase difference was plotted against the network cycle period for each preparation ($n = 131$). Each point represents the average over at least 12 cycles. Errors bars demonstrate the regular cycle period and G3 to G4 phase difference in each preparation. C: the oscillator interneurons in G3 were more effective in determining the phase of the G2 coordinating interneurons than were the oscillator interneurons in G4. The phase of the G2 coordinating interneurons were held more tightly to the G3 than the G4 oscillator interneurons. The G2 to G3 (○) and G2 to G4 (●) phase differences were plotted against the G3 to G4 phase difference ($n = 34$). Regression lines are indicated as a solid (G2 to G4) or a dotted (G2 to G3) line. See text for further explanation. D: duty cycles of the G2 coordinating interneurons were shorter than predicted. The solid line represents the expected duty cycles over a range of G3 to G4 phase differences if the G2 coordinating interneurons were active for the entire time the ipsilateral G3 and G4 oscillator interneurons were silent. The maximum expected duty cycle was ~43% when the G3 to G4 phase difference was 0%. The expected time available for the coordinating interneurons to burst decreased as the G3 to G4 phase difference increased, regardless of which oscillator led.
tions but potentially by modulation of intrinsic membrane properties or synaptic connections within the network. One method to generate phase differences is proposed by the excitability-gradient hypothesis, which states that inherent period differences between symmetrically coupled segmental oscillators produce intersegmental phase differences (Grillner et al. 1993). This hypothesis has been tested and rejected in a variety of coupled segmental oscillator pattern generating networks (leech swimming, Friesen and Pearce 1993; lamprey swimming, Cohen 1987a; Sigvardt 1993; Sigvardt and Williams 1996). In the following paper, the excitability-gradient hypothesis is explored fully in modeling studies using both symmetric and asymmetric versions of the heartbeat timing network (Hill et al. 2002).

The flexibility in phase relationships observed in the heartbeat timing network not only contrasts with the constancy observed in other intersegmental central pattern generators but also begs the question of the functional significance of these differences. The oscillator heart interneurons of the leech heartbeat timing network are after all premotor interneurons that establish motor output via their inhibitory connections with heart motor neurons, which in turn directly excite the muscle cells that form the lateral heart tubes (Thompson and Stent 1976a,b). Previous observation (Thompson and Stent 1976a,b) and more recent quantitative study (Wenning et al. 2000) indicate that the pattern of motor outflow to the hearts, measured in the isolated intact nerve cords, albeit bilaterally asymmetric, is intersegmentally quite regular. Thus examination of less reduced preparations may provide insight into the significance of the G3 to G4 phase relationships observed here.

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