Cycle Period of a Network Oscillator Is Independent of Membrane Potential and Spiking Activity in Individual Central Pattern Generator Neurons

Paul S. Katz, Akira Sakurai, Stefan Clemens, and Deron Davis

Department of Biology, Georgia State University, Atlanta, Georgia 30303-3088

Submitted 4 September 2003; accepted in final form 26 April 2004

Katz, Paul S., Akira Sakurai, Stefan Clemens, and Deron Davis. Cycle period of a network oscillator is independent of membrane potential and spiking activity in individual central pattern generator neurons. J Neurophysiol 92: 1904–1917, 2004. First published April 28, 2004; 10.1152/jn.00864.2003. Rhythmic motor patterns are thought to arise through the cellular properties and synaptic interactions of neurons in central pattern generator (CPG) circuits. Yet, when examining the CPG underlying the rhythmic escape response of the opisthobranch mollusc, Tritonia diomedea, we found that the cycle period of the fictive swim motor pattern recorded from the isolated nervous system was not altered by changing the resting membrane potential or the level of spiking activity of any of the 3 known CPG cell types: ventral swim interneuron-B (VSI-B), the dorsal swim interneurons (DSIs), and cerebral neuron 2 (C2). Furthermore, tonic firing in one or more DSIs or C2 evoked rhythmic bursting that did not differ from the cycle period of the motor pattern evoked by nerve stimulation, regardless of the firing frequency. In contrast, the CPG produced a large range of cycle periods as a function of temperature. The temperature sensitivity of the fictive motor pattern produced by the isolated nervous system was similar to the temperature sensitivity of the swimming behavior produced by the intact animal. Thus, although the CPG is capable of producing a wide range of cycle periods under the influence of temperature, the membrane potentials and spiking activity of the identified CPG neurons do not determine the periodicity of the motor pattern. This suggests that the timing of activity in this network oscillator may be determined by a mechanism that is independent of the membrane potentials and spike rate of its constituent neurons.

INTRODUCTION

Understanding the mechanisms for producing periodic neuronal activity has important implications for sensory processing (Perez-Orive et al. 2002), thalamo-cortical function (Steriade 1999), and motor control (Calabrese 1995). A considerable amount is known about the mechanisms underlying cellular membrane potential oscillators (see Benson and Adams 1987; Jacklet 1989), but much less is understood about the mechanisms that underlie the generation of rhythmic activity by so-called network oscillators, which produce rhythmic patterns of activity as an emergent property of the interactions of neurons that lack intrinsic oscillatory properties (for examples, see Jacklet 1989). In some network oscillators, such as half-center oscillators, it has been shown that the spiking properties and the membrane potentials of component neurons help determine the periodicity of the oscillations (Calabrese 1995; Marder and Calabrese 1996). We tested whether such factors affect the periodicity of a slightly more complex network oscillator, the central pattern generator (CPG) underlying the escape swim CPG of the mollusc, Tritonia diomedea.

The Tritonia escape swim is a stereotyped fixed action pattern (Getting 1989a; Willows and Hoyle 1969). A single episode of escape swimming lasts up to a minute and consists of a series of alternating dorsal/ventral whole body flexions. It has been proposed that the Tritonia swim CPG is a pure network oscillator in which none of the component neurons exhibits intrinsic oscillatory activity (Getting 1989a). Rhythmic activity has been proposed to be produced through a combination of reciprocal inhibition combined with delayed excitation among the 3 CPG cell types (Getting 1989a).

Computer simulations of the CPG suggest that the cycle period is strongly influenced by the time course of synaptic potentials and spiking properties such as spike frequency adaptation (Getting 1983a, 1989b). However, previous results indicated that the periodicity of the motor pattern is not determined by the level of spiking activity in some CPG neurons (Fickbohm and Katz 2000). Here we more thoroughly investigate whether the membrane potential or activity levels of the CPG neurons play a role in determining the cycle frequency of the swim motor pattern.

We found that the cycle period is insensitive to the level of activity exhibited by individual neurons in the CPG, but that the Tritonia escape swim motor pattern can exhibit a range of cycle periods when the nervous system is exposed to different temperatures. These results have important implications for the mechanisms governing motor pattern generation in this model network oscillator.

Portions of this work were presented previously in abstract form (Katz et al. 2002).

METHODS

Animals

Tritonia diomedea were obtained from Living Elements Ltd. (Delta, B.C., Canada) and maintained in recirculating artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) at 11°C.

Electrophysiology

The Tritonia brain, composed of the fused cerebropleural and pedal ganglia, was dissected from the animal as previously described (Fickbohm and Katz 2000), dipped in 0.5% glutaraldehyde for 20–30 s to immobilize contractile elements in the ganglionic sheath, and pinned dorsal side up in a 35-mm petri dish lined with Sylgard (Dow Corning, Midland, MI). The ganglionic sheath was then mechanically removed.
with forceps and scissors. Normal saline, consisting of (in mM) 420 NaCl, 10 KCl, 10 CaCl$_2$, 50 MgCl$_2$, 10 d-glucose, and 10 HEPES, pH 7.6, was continuously superfused over the ganglia using a gravity flow system at a rate of about 1 ml/min. The saline was chilled before entry into the petri dish by running the polyethylene inflow tube through a Plexiglas chamber surrounding the petri dish atop a Peltier cold plate. Temperature was monitored with a flexible probe (Physitemp, Clifton, NJ) positioned close to the ganglion in the dish and adjusted by changing the voltage to the Peltier device.

Intracellular recordings were obtained with glass microelectrodes (1–15 MΩ) filled with 3 M KCl. Signals were recorded using Axoclamp 2B amplifiers (Axon Instruments, Union City, CA) and digitized with a 1401plus A/D system and analyzed with Spike2 software (both from Cambridge Electronic Design, Cambridge, UK). To monitor and trigger the swim motor program, suction electrodes were placed on pedal nerve 3 (PDN3), PDN5, and PDN6 (Willows et al. 1973). Fictive swim motor programs were elicited by unilaterally stimulating (2 ms, 10 to 20 V pulses at 10 to 20 Hz for 1 s) PDN3, which contains the axons of sensory neurons.

The pathway for eliciting a swim motor pattern is illustrated in Fig. 1A. Sensory neurons converge on a trigger interneuron (TrI) and a commandlike neuron, the dorsal ramp interneuron (DRI) (Frost and Katz 1996; Frost et al. 2001). DRI synapses on the dorsal swim interneurons (DSIs). The DSIs, together with the ventral swim interneurons (VSI-A, VSI-B) and interneuron C2 (cerebral neuron 2) form the central pattern generator (CPG) that produces the swim motor pattern (Getting 1989a). All neurons are bilaterally represented. The CPG neurons synapse on efferent flexion neurons: the ventral flexion neurons (VFN) and the dorsal flexion neurons (DFN-A, DFN-B).

FIG. 1. Depolarization of VSI-B did not affect the periodicity of the swim motor pattern. A: schematic diagram showing the swim central pattern generator (CPG) and its inputs. Inhibitory synapses are represented as filled circles, excitatory synapses are shown as triangles, and mixed synapses are combinations (Getting 1981). Dotted lines represent polysynaptic pathways. B and C: simultaneous intracellular recordings from 2 efferent CPG follower neurons [a dorsal flexion neuron (DFN) and ventral flexion neuron (VFN)], 2 CPG neurons [dorsal swim interneuron (DSI) and ventral swim interneuron-B (VSI-B)] and an extracellular recording of pedal nerve 5 (PDN5). Initial resting potentials were −60, −55, −60, and −50 mV, respectively. B: stimulation of pedal nerve 3 (solid arrow: 10 V, 15 pulses, 10 Hz) triggered 5 burst cycles with DSI and VSI-B firing in alternation. C: injection of constant depolarizing current (2 nA, beginning at open arrow) caused VSI-B to fire tonically, but did not affect the periodicity of the motor pattern triggered by nerve stimulation (solid arrow). D: DSI spike times from B and C show that the initial period (T) was unaffected by injecting current into VSI-B. T was defined as the time period between the mean spike times (arrowheads) of the first 2 bursts.

There are 2 types of VSI: VSI-A and VSI-B. VSI-A does not appear to play a significant role in the production of the motor pattern, whereas VSI-B is a necessary component of the CPG (Getting 1983b, 1989a). Therefore VSI-A was not examined here except as a monitor of the swim motor pattern.

There are 3 DSIs in each hemisphere of the cerebral ganglion: DSI-A, DSI-B, and DSI-C (Getting et al. 1980). DSI-B and DSI-C are completely equivalent neurons and differ from DSI-A only slightly in their connectivity (Getting 1981). It is difficult to classify the DSIs without recording from all 3, so when we recorded from multiple DSIs, they were assigned an arbitrary number.

Current was injected into the somata of individual neurons through a balanced bridge circuit. Different amounts of positive and negative current were injected in different experiments in the range of 0.5 to 16 nA. The amount of current used in each experiment was dependent on the properties of the electrode and the cell. The maximum amount of current that could be injected without injuring the neuron was used. In all cases, the current had a substantial effect on the activity of the injected neuron; depolarizing current always evoked strong spiking responses. The precise extent of hyperpolarization produced in each case is not known because a bridge circuit was used. However, based on the input resistances of the neurons [C2: 13 MΩ; DSI: 31 MΩ; VSI: 15 MΩ (Getting 1983a, 1989b)], it is expected that even with the minimum negative current used (−1 nA), the resulting hyperpolarization of
the membrane potential in the soma should be around $-13$ mV. In most cases, the hyperpolarization was substantially greater.

To elicit tonic spiking, repeated 20 ms, 7 to 12 nA current pulses were injected at a set periodicity (5–20 Hz). Each current pulse elicited a single action potential. The success of stimulation was monitored either by observing the peak waveform of the action potentials, by observing the spike afterhyperpolarizations, or by recording one-for-one excitatory postsynaptic potentials (EPSPs) in follower neurons.

Swim cycle analysis

The cycle period progressively lengthens during the course of a single swim episode, starting around 7–8 s and ending around 10–12 s (Lennard et al. 1980). Therefore to compare motor patterns, which had varying numbers of cycles, we measured the initial cycle period (T). This was calculated as the time interval between the mean spike times of the first 2 complete bursts after the onset of the motor pattern (see Fig. 1D). The mean spike time was chosen because it is a more reliable marker than either the first or last spike. C2 was often used as the monitor of bursting because its bursts are the most regular, although equivalent results were obtained from any of the CPG neurons or the extracellular nerve recording.

All quantitative results are expressed as means ± SE. Paired t-tests were used to compare the effects of depolarization and hyperpolarization on the swim period.

Behavioral experiments

Behavioral data were obtained from 3 animals treated in parallel over a 3-week period. To evoke the escape swim behavior, animals were stimulated with 5 ml of a 5 M NaCl salt solution applied by use of a syringe over the posterior region of the body surface. The resulting swimming response was recorded by video camera and analyzed for cycle period. Animals were videotaped and the time was recorded on the tape with a date/time generator (WJ 810, Panasonic).

A single cycle was defined as the time from one maximum ventral flexion until the next. Animals were stimulated 4 times at each test temperature at 10-min intervals. After each animal was tested at a given temperature, the tank temperature was adjusted in increments of 1°C. The animals were allowed 2.5 h to equilibrate to the new temperature.

Results

To determine whether the periodicity of the Tritonia swim motor pattern is controlled by the membrane potentials or spiking activities of the known CPG neurons, we examined the effects of biasing the membrane potentials of individual CPG neurons using tonic current injection into the cell body. It is not possible to record from all 10 CPG neurons simultaneously (2 VSIs, 6 DSIs, and 2 C2s); therefore we examined the effects of current injection on each cell type in the CPG. We also examined the effect of tonic action potential firing produced by repeatedly injecting short current pulses.

Cycle period was not affected by manipulating VSI-B membrane potential

There is a single VSI-B on each side of the brain. We tested the effect of changing VSI-B firing on the periodicity of the fictive swim motor pattern. A typical pattern of bursting activity was recorded intracellularly in CPG neurons, efferent flexion neurons, and extracellularly on pedal nerves in response to electrical stimulation of a body wall nerve (at arrow, Fig. 1B). VSI-B inhibits the other CPG members (see Fig. 1A) and strong tonic firing of a single VSI-B is sufficient to inhibit rhythmic motor pattern production (A. Sakurai, unpublished observation). A lower level of depolarizing current injected into VSI-B (here: 2 nA) caused the normally quiescent VSI-B to fire tonically, but did not suppress the motor pattern (Fig. 1C). The initial cycle period (T), as indicated by the DSI spike times, was not affected by the constant VSI-B firing (Fig. 1D).

The participation of the VSI-Bs is necessary for motor pattern generation: hyperpolarization of both VSI-Bs generally prevents the production of the swim motor pattern (Getting 1983b). Suppressing the firing of a single VSI-B with hyperpolarizing current injection did not halt motor pattern production, allowing us to study the effect of VSI-B activity on the rhythmicity of the motor pattern (Fig. 2).

Beginning before the nerve stimulus, constant current (–7 nA) was injected into VSI-B, causing the membrane potential to hyperpolarize more than 50 mV (Fig. 2B, open arrow; Fig. 2C, red trace). Nerve stimulation caused normal bursting in the other CPG neurons; however, spiking in VSI-B was greatly attenuated and full spikes were completely absent from most of the cycles. Despite the reduction in VSI-B spiking, the initial cycle period was unaffected, as indicated by the C2 spike times (Fig. 2D).

On average, injection of depolarizing current (0.5 to 2 nA) or hyperpolarizing current (–2 to –16 nA) into VSI-B had no significant effect on the initial cycle period until the motor pattern was completely blocked (Fig. 2E). The average initial period for all preparations without current injection was 8.4 ± 0.5 s (n = 14). The average initial period with depolarizing current injected into VSI-B for those cases where bursting was not blocked was 9.1 ± 1.0 s, which was not statistically different from control values for those preparations (P = 0.7, n = 5, paired t-test). The average initial period for preparations in which one VSI-B was hyperpolarized was 8.0 ± 0.7 s, which was not significantly different from control values for those preparations (P = 0.2, n = 10, paired t-test). In 4 preparations, injection of hyperpolarizing current (–5 to –6 nA) into both left and right VSI-Bs did not halt the swim motor pattern and resulted in a mean initial period of 9.8 ± 0.4 s, which was not significantly different from control for those preparations (P = 0.2, n = 4, paired t-test). Thus although injecting current into VSI-B could completely disrupt the motor pattern, in preparations where hyperpolarization of VSI-B did not prevent production of the motor pattern, there was no effect on the initial cycle period.

Cycle period was not affected by manipulating DSI membrane potential

There are 6 DSIs, 3 on each side of the brain. We were able to inject current (1 to 5 nA) simultaneously into 2 or 3 of the 6 neurons to bias their membrane potentials either in the depolarizing or hyperpolarizing direction while simultaneously recording from CPG neuron C2 (Fig. 3). Tonic depolarization of the DSIs caused them to increase their rate of spiking before initiation of the swim motor program (Fig. 3B, open arrows). However, the initial cycle period was unaffected, as indicated by the mean spike times of the C2 bursts (Fig. 3D).

It was previously reported that hyperpolarizing multiple DSIs could block rhythmic activity altogether (Getting and...
However, in preparations in which hyperpolarization of 2 or 3 DSIs was not sufficient to prevent rhythmic motor pattern production, it had no effect on the initial cycle period, although it strongly reduced the DSI firing during the swim motor program and reduced the number of cycles (Fig. 3, C and D).

On average, the initial cycle period was not significantly affected by either depolarization or hyperpolarization of 2 or 3 DSIs (Fig. 3E). The average initial period under control conditions was 6.7 ± 0.6 s (n = 6). With the DSIs depolarized, the initial period was 6.9 ± 0.1 s, which was not a significant change from control values in those preparations (P = 0.5, n = 3, paired t-test). When the DSIs were hyperpolarized, the initial period was 6.7 ± 0.7 s, which also was not a significant change from control in those preparations (P = 0.8, n = 5, paired t-test). Thus biasing the membrane potentials of 2 or 3 DSIs did not alter the periodicity of the motor pattern, until it exceeded a certain level, at which point the motor pattern was completely suppressed (Getting and Dekin 1985).

**Tonic DSI firing elicited bursting of the same periodicity as the nerve-evoked swim motor pattern**

The DSIs are the only known targets of the commandlike neuron DRI (see Fig. 1A) (Frost and Katz 1996) and tonic activation of the DSIs has been shown to elicit a swim motor pattern in some preparations (Fickbohm and Katz 2000; Frost et al. 2001). In the set of experiments reported here, tonic DSI stimulation elicited rhythmic bursting activity in other CPG neurons in 20 out of 27 preparations tested. The spike frequency required to elicit bursting varied between preparations. Increasing the spike frequency or the number of DSIs firing sometimes allowed the bursting to occur (Fig. 4A). In the example shown in Fig. 4A, when one DSI was stimulated at 5
Hz, it caused a slight depolarization of the membrane potential of C2 and a slight inhibition of the firing in the other DSIs. Adding a second DSI at 5 Hz further excited C2, causing it to spike. The addition of a third DSI at 5 Hz caused C2 and other neurons recorded on PDN6 to fire action potentials in a rhythmic bursting pattern.

Bursting elicited by tonic DSI firing had a consistent cycle period, regardless of the frequency of DSI firing. In the example from Fig. 4A, increasing the spike frequency of the DSIs to 10 Hz and then to 20 Hz did not change the cycle period of the bursting. The periodicity of the bursting pattern evoked by tonic stimulation of the DSIs was similar to that of the nerve-evoked swim (Fig. 4, B–F). The nerve-evoked motor pattern was recorded extracellularly (PDN6) and intracellularly in C2 and 3 DSIs (Fig. 4B). When one of the DSIs was stimulated to fire at 10 Hz (Fig. 4C), it evoked bursting in C2 and on PDN6 that was similar in period to that evoked by nerve stimulation (compare C2 spike times in Fig. 4E). Stimulating 2 DSIs simultaneously at 20 Hz produced bursting in C2 at the same periodicity (Fig. 4, D and E). In 2 preparations, we tested 3 DSIs at 20 Hz and again found no consistent or significant difference in the periodicity compared with the nerve-evoked swim.

For all of the preparations examined, there was no significant difference in the initial period of bursting, regardless of the number of DSIs stimulated or their spike frequency (Fig. 4F). The average initial cycle period in response to nerve stimulation in these preparations was $6.9 \pm 0.4$ s ($n = 9$). When a single DSI was stimulated to fire at 10 or 20 Hz, the average initial period was $7.7 \pm 0.5$ and $6.8 \pm 0.6$ s, respectively, neither of which was significantly different from control ($n = 4$, $P = 0.6$ and 0.5, respectively, paired t-test). Stimulating 2 DSIs at 10 or 20 Hz did not significantly affect the initial cycle period ($n = 4$, mean values of $7.2 \pm 0.5$ and $6.3 \pm 0.4$, $P$ values of 0.8 and 0.7, respectively). Thus although spiking in the DSIs could elicit bursting activity, the level of spiking activity in the DSIs did not determine the periodicity.

**Cycle period was not affected by manipulating C2 membrane potential**

Biasing the membrane potential of a single C2 in either depolarizing or hyperpolarizing direction altered the firing in that cell but did not affect the periodicity of the nerve-evoked motor pattern (Fig. 5). Injection of a maintained constant depolarizing current (1 to 5 nA) into one C2 caused it to begin firing tonically before nerve stimulation (Fig. 5B, open arrow) but did not affect the periodicity of bursting in the other CPG neurons during a nerve-evoked swim motor pattern (beginning at the solid arrow). In the same preparation, injection of hyperpolarizing current ($-1$ to $-5$ nA) into one C2 also did not significantly affect the burst period (Fig. 5C). Although C2 started from a more hyperpolarized potential, it rapidly depolarized to spike and remained depolarized throughout the motor pattern (Fig. 5C), resulting in a larger voltage excursion (Fig. 5D). The initial cycle period measured from the spike times of the contralateral C2 was unaffected by any level of current injection (Fig. 5E).

On average, changing the membrane potential of a single C2 did not have a significant effect on the initial cycle period (Fig. 5F). The average initial period under control conditions was $7.2 \pm 0.5$ s ($n = 9$). When C2 was depolarized, the initial
period was 7.3 ± 0.6, which was not significantly different from control values in those preparations (P = 0.4, n = 6, paired t-test). When C2 was hyperpolarized, the average initial period was 7.5 ± 0.7, which was not significantly different from control values in those preparations (P = 0.4, n = 7). Thus biasing the membrane potential of a single C2 did not alter cycle period.

As with VSI-B, there is only a single C2 on each side of the brain. C2 is a necessary component of the CPG; hyperpolarization of both the left and right C2s with injection of as little as −0.5 nA prevented production of the swim motor pattern as previously reported (Getting and Dekin 1985). In the example shown in Fig. 6, A–C, −1 nA injected into both C2s prevented the swim motor pattern (Fig. 6B). However, injection of −1 nA into just a single C2 did not alter the periodicity of the motor pattern, although it decreased the burst duration of the injected cell and increased the amplitude of its membrane potential oscillations (Fig. 6C).

In one preparation, we were able to systematically increase the amount of hyperpolarizing current injected into both C2s without failure of the motor pattern (Fig. 6D). In this preparation, injection of −5 nA into both C2s was sufficient to suppress rhythmic bursting (Fig. 6Divi). However, injection of −3 nA into a single C2 (Fig. 6Dii) or both C2s (Fig. 6Div) did not change the initial cycle period, although it decreased the number of cycles.

C2-triggered motor patterns had the same periodicity as nerve-evoked motor patterns

It was previously reported that brief depolarization of C2 is sufficient to trigger a swim motor pattern (Taghert and Willows 1978). We observed this only in a minority of preparations (7 out of 28 in this study). However, when it occurred, the periodicity of the motor pattern evoked in this fashion was very similar to that caused by nerve stimulation (Fig. 7). Although self-sustained motor patterns caused by brief C2 stimulation were rarely observed, it was previously reported that maintained current injection into C2 also can elicit a swim motor pattern for as long as C2 is depolarized (Getting 1977). We obtained this response in 2 preparations and observed that the periodicity of the swim motor pattern produced in this manner

---

**FIG. 4.** Tonic DSI firing produced a motor pattern with the same period as the nerve-evoked pattern. A–D: simultaneous intracellular recordings from C2 and 3 ipsilateral DSIs, and an extracellular recording from PDN6. A: 3 DSIs were stimulated to fire at defined frequencies by injection of repeated 20 ms, 10-nA current pulses that each evoked a single spike. Progressively increasing the frequency of DSI firing and stimulating more DSIs did not affect the periodicity of C2 bursting. B: typical swim motor pattern was evoked in response to stimulation of pedal nerve 3 (PDN3, 5 V, 10 Hz, 0.5 s at arrow). C: DSI-3 was stimulated to fire at 10 Hz, evoking a typical bursting pattern in C2 and causing hyperpolarization of the other 2 DSIs attributed to recruitment of inhibition (Getting and Dekin 1985). D: similar pattern of bursting in C2 was elicited when both DSI-2 and DSI-3 were stimulated to fire at 20 Hz. As in C, DSI-1 was initially inhibited by the DSI stimulation, but showed bursting in subsequent cycles. E: C2 spike times from B–D show that the periods are similar despite the large differences in the amount of DSI firing. Triangles and bars above each trace represent the first cycle period in each trace as determined by the mean spike times. F: average initial burst period was not affected by a range of DSI tonic firing. See text for details.
was also similar to that caused by nerve stimulation (data not shown). Thus the periodicity of the motor pattern was the same when triggered by C2 depolarization or by stimulation of the afferent axons in the body wall nerve.

**Tonic C2 firing elicited bursting of the same periodicity as the nerve-evoked swim motor pattern**

In some preparations, we were able to elicit rhythmic bursting in CPG neurons when C2 was stimulated to fire tonically, using repetitive 20-ms current pulses (5 to 12 nA at either 10 or 20 Hz). Tonic firing in C2 caused rhythmic activity in 6 of 11 preparations where it was tested (Fig. 8). In each case, the periodicity of the motor pattern elicited by tonic C2 firing was similar to that caused by nerve stimulation regardless of the frequency of C2 spiking (Fig. 8, C and D). Thus the periodicity of the motor pattern was not dependent on the level of spiking activity exhibited by a single C2.

The ability of tonic C2 firing to elicit bursting activity was not as robust as that of the DSIs. It was often not possible for C2 tonic firing to elicit bursting repeatedly in a single preparation. In one preparation, neither tonic C2 firing alone (Fig. 9B) nor tonic DSI firing alone (Fig. 9C, initial 10 s of trace) elicited rhythmic activity. However, combining tonic C2 firing with tonic firing in 2 DSIs caused rhythmic activity at a similar periodicity to the nerve evoked swim (Fig. 9, A, C, and D). Thus the periodicity of the bursting activity was consistent even in preparations that required more tonic spiking activity to produce it.

**Temperature sensitivity of the swim motor pattern**

None of the manipulations of CPG neuron activity in this study caused any change in the periodicity of the swim motor pattern. It is possible that, unlike other known CPGs, this oscillator might be capable of operating at only one cycle period. In an effort to see whether the CPG was capable of producing different cycle periods, we tested its performance at varying temperatures.

We found that the ability to evoke a swim motor pattern was temperature sensitive. The percentage of nerve stimuli that failed to evoke a swim motor pattern increased at the extremes
of the temperature range tested (6–20°C). However, if a swim motor pattern was evoked, then the cycle period of the swim motor pattern was strongly temperature dependent (Fig. 10). At 5°C, the initial cycle period in the example shown in Fig. 10A was 11.9 s (see also Fig. 10D). It decreased to 7.2 s at 10°C (Fig. 10, B and D), and decreased further to 4.2 s at 15°C (Fig. 10C).

**FIG. 6.** Cycle period was not affected by changing the membrane potentials of both C2s. A–C: simultaneous intracellular recordings from a right DSI, both left and right C2s, and an extracellular recording of activity on the right pedal nerve 3 (R-PDN3). Initial resting potentials were −60, −50, and −60 mV, respectively. A: 3 burst cycles were evoked by stimulation of the L-PDN3 (20 V, 10 Hz, 1 s). B: when both C2s were hyperpolarized with −1 nA constant current, then the same nerve stimulus did not evoke rhythmic bursting activity. C: injection of −1 nA into just the right C2 did not affect the initial burst period. D: spike times of a DSI are shown in response to nerve stimulation. Each line represents a different trial (i–vii). Trials were separated by 5 min or more. Current was injected into one or both C2s as indicated. When both C2s were hyperpolarized with −5 nA (vii), the DSI produced a single burst, the subsequent spiking was not organized into a burst, and thus the cycle period could not be measured. Trials shown in D are from a different preparation than those in A–C.

**FIG. 7.** Brief depolarization of C2 elicited rhythmic bursting at a periodicity similar to the nerve-evoked swim motor pattern. A: stimulation of PDN3 (at arrow) produced a typical 3-cycle swim motor pattern as recorded intracellularly in left and right DSIIs and C2 interneurons. B: in the same preparation, brief depolarization of the right C2 (2 nA for 2 s between up and down arrows) triggered a bout of repetitive bursting in the CPG neurons that strongly resembled the nerve-evoked swim motor pattern and had a similar periodicity. C: spike times of the L-C2 from the examples in A and B. Initial period, highlighted by the downward triangles, is 7.8 s for the nerve-evoked swim motor pattern and 8.0 s for the C2-triggered pattern. D: average initial period of the nerve-evoked motor pattern (6.7 ± 0.4 s) is not significantly different from the initial period of the C2-triggered swim (7.3 ± 0.4 s, n = 4, P = 0.14, paired t-test).
The average initial cycle period over the whole temperature range for all preparations tested is shown in Fig. 10E. There was a progressive decrease in cycle period with increasing temperature. Thus, unlike changes to the membrane potentials or spiking levels of the CPG neurons, varying the temperature profoundly affected the periodicity of the motor pattern.

The temperature sensitivity of the cycle period of the swim motor pattern evoked by nerve stimulation was similar to that of the swimming behavior produced by the intact animal (Fig. 8).

FIG. 8. Tonic C2 firing elicited bursting at the same periodicity as the nerve-evoked swim motor pattern. A: typical motor pattern was recorded extracellularly on PDN6 and intracellularly in a right DSI (R-DSI) and both right and left C2s (R-C2, L-C2) in response to electrical stimulation of the left PDN3 (5 V, 10 Hz, 0.5 s at arrow). B: in the same preparation, C2 was made to fire tonically at 10 Hz by injection of 20-ms current pulses. This elicited bursting in PDN6, the DSI, and the other C2. Note that the underlying membrane potential of the left C2 exhibited membrane potential oscillations despite the tonic firing. C: spike times of the right C2 show that the periodicity of the motor patterns in A and B are similar. D: average initial periods from motor patterns evoked by nerve stimulation were not significantly different from when evoked by tonic C2 stimulation. Mean initial period for nerve-evoked swims was 6.2 ± 0.4 s (n = 4). When C2 was stimulated to fire tonically at 10 Hz, the mean initial period was 6.2 ± 0.3 s (n = 3) and when it was stimulated to fire at 20 Hz, the mean initial period was 6.5 ± 0.2 s (n = 2). There was no significant difference between the initial period of nerve-evoked motor patterns and those evoked by C2 stimulation (n = 4 preparations, P = 0.52, paired t-test). Results from preparations stimulated at either frequency were pooled. Some, but not all, preparations were tested at both frequencies.

FIG. 9. Combining C2 and DSI stimulation elicited bursting at the same periodicity as the nerve-evoked swim motor pattern. A: swim motor pattern was elicited by stimulating PDN3 (arrow). Rhythmic bursting was recorded extracellularly on PDN6 and intracellularly in both C2s and 2 contralateral DSIs. B: tonic firing in the left C2, produced by 20-ms current pulses repeated at 10 Hz did not produce rhythmic bursting in this preparation. There was a slight synaptic effect on the contralateral C2 and on the 2 DSIs. Thickening of the bottom trace is attributed to the stimulus artifacts. C: when both DSIs were made to fire at 20 Hz using brief current pulses (20 ms), no rhythmic bursting was recorded in either of the C2 neurons or on PDN6. Additional tonic firing in the left C2 at 10 Hz caused bursting activity in the right C2 and on PDN6. Erratic changes in the baseline membrane potential of the R-DSI are probably attributable to changes in the electrode resistance affecting the bridge balance. D: spike times from the right C2 show that the periodicity of the bursts were similar in the nerve-evoked motor pattern (A) and with tonic stimulation of C2 and the DSIs (C).
FIG. 10. Cycle period was temperature sensitive. A–C: a swim motor pattern was elicited by stimulating pedal nerve 2 (PDN2, at arrows: 20 V, 10 Hz, 1.5 s). Rhythmic activity was recorded extracellularly on PDN6 and intracellularly in a DSI and a C2. Temperature was varied in vitro by cooling the inflow solution with a Peltier device. A: at 5°C, nerve stimulation evoked 3 burst cycles, which were complete within 37 s. B: at 10°C, the same stimulus evoked 4 cycles with a shorter burst period. C: at 15°C, nerve stimulation again produced 3 cycles, but the total duration of the episode decreased to 11 s. Traces are not shown in temporal order; the preparation was initially cooled to 5°C and warmed to 10°C within 2.5 min. It was then further warmed and the recording at 15°C was made 13 min after the 10°C recording. D: initial cycle period, as measured by C2 spike times, decreased with increasing temperature. E: average initial cycle period of the swim motor pattern decreased as temperature increased. Each bar represents the mean ± SE of the initial cycle period for the temperature ranges shown. Numbers in each bar indicate the number of trials averaged. Not all temperatures were tested in each preparation. Average initial periods showed significant changes as determined by a one-way repeated-measures ANOVA with multiple pairwise comparisons (Tukey test). All of the temperature ranges exhibited significant pairwise differences from the others (P < 0.05) except 12–15°C. (ii) compared with 9–12°C (i) or 15–18°C (iv) compared with 18–21°C (v), which were not significantly different. F: initial cycle period of the swimming behavior of the intact animal exhibited a similar dependency on temperature. Three animals were tested in parallel. Four trials were conducted for each animal at each temperature. All temperature ranges (i–iii) were significantly different (P < 0.05) from the others, as judged by a one-way repeated-measures ANOVA with multiple pairwise comparisons (Tukey test).

In the temperature range of 9–12°C, the average period of the initial flexion of a swim, measured from the time of the first full ventral body flexion to the peak of the next ventral flexion, was 7.1 ± 0.2 s (Fig. 10F, bar ii). When the temperature was within the range of 12–15°C, the initial cycle period decreased to 5.6 ± 0.2 s (bar iii). Conversely, when the temperature was lowered to 6–9°C, the initial cycle period increased to 10.2 ± 0.6 s (bar i). These differences in the cycle periods of the behaviors in each temperature range are statistically significant (see figure legend). The extent of change exhibited by the behavior was similar to that of the isolated nervous system (Fig. 10, E and F).

Although the cycle period of the motor pattern was greatly affected by temperature, the percentage of each period that the neurons were active remained constant. The burst duration of the swim interneurons increased as the period increased (Fig. 10, A–C, Fig. 11). In addition, the phasing between dorsal and ventral phase interneurons was unaffected by temperature (Fig. 11). Thus the shapes of the bursts and the phasing of the components did not differ despite the large difference in cycle period.

In one isolated-brain preparation, we were able to test whether the periodicity of bursting elicited by C2 varied with temperature. We found that the burst period evoked by C2 firing at 20 Hz was similar to that of the nerve-evoked swim at each temperature where it was tested (data not shown). The initial period of the nerve-evoked motor pattern was 7.1 s at 10°C and increased to 10.8 s at 7°C. The initial burst period when C2 was stimulated to fire at 20 Hz was 6.7 s at 10°C and increased to 10.2 s at 7°C. Thus the effect of temperature on cycle period was intrinsic to the CPG and not attributed to a change in the inputs.

DISCUSSION

The Tritonia swim CPG has been described as a network oscillator in which the rhythmic motor pattern is produced through the interactions of neurons that lack bursting properties
in isolation (Getting 1989a). In an effort to better understand motor pattern generation in this model system, we manipulated the membrane potentials and spiking activity of component neurons in the CPG. To our surprise, we found that manipulating the membrane potentials or levels of spiking activity of CPG interneurons over a wide range of values did not affect the periodicity of the swim motor pattern.

Our expectation was that the period of oscillation would be influenced by the level of activity of the CPG neurons. For example, one simple scenario is that if the membrane potentials of the CPG neurons were closer to threshold or, indeed, above threshold, then the period ought to decrease because it would take less time to begin the next cycle, whereas if the membrane potentials were farther from threshold, then it should take longer to depolarize and thus increase the period. Or alternatively, depolarizing a neuron might have increased the period by increasing the inhibition on other neurons. However, when any of the CPG neurons, even the normally silent C2 and VSI-B, was depolarized to the point of spiking, the cycle period was unaffected. Conversely, when any of these neurons was hyperpolarized, it did not slow down the motor pattern, but merely caused the hyperpolarized neuron to exhibit a greater voltage excursion during the bursts (Fig. 2C, Fig. 5D). These results suggest either that the circuit can compensate for differences in activity of component neurons or that activity levels of these neurons do not play important roles in determining the periodicity of the motor pattern.

It is possible that current injection into the cell body does not affect the membrane potential at important integrative areas that are electrically distant from the soma. Previous work suggested that the length constant for the DSIs was about 2 mm (Getting 1983a), which is approximately the distance from the soma to the farthest point in the pedal ganglion. Therefore based on this measurement, even if the synapses were located as distant as physically possible, one would expect a maximum voltage attenuation of 36% of the value in the soma. Thus a −50 mV hyperpolarization in the soma would still produce a substantial −18 mV change in these distant sites. The ability to affect distant sites in the neuron using current injection into the soma is further suggested by the fact that hyperpolarization of the neurons decreased or eliminated spiking in all cases. Furthermore, hyperpolarization of both C2s with as little as −0.5 nA of current was able to suppress the motor pattern, whereas higher levels of current injected into a single C2 had no effect on periodicity. Thus the lack of an effect of current injection on cycle period cannot be attributed to an inability to alter the membrane potential at distant synapses.

The inability to prevent motor pattern generation by strong hyperpolarization of a single C2 or VSI-B indicates that the contralateral counterpart is capable of maintaining the rhythm without its partner. Thus although activity in at least one cell is necessary for motor pattern generation, altering the membrane potential of either cell did not affect the periodicity, whereas hyperpolarizing both cells often caused the pattern to cease.

The periodicity was not affected by the spike frequency in DSI or C2 when these neurons were driven to fire at defined frequencies. With the DSIs, we were able to stimulate over a wide range of frequencies (5–20 Hz) and include one to 3 neurons, effectively producing a 12-fold change in the total number of spikes per second. Regardless of the DSI tonic spike frequency, the cycle period of the resulting swim motor pattern was consistent. Thus the cycle period was independent of the level of spiking activity in DSI or C2. This suggests further that the periodicity is not controlled by the sensory or command inputs to the CPG since stimulating the DSIs and C2 bypasses the input pathway (Fig. 1A).

**A permissive role for DSI and C2 in rhythmogenesis**

DSI and C2 are necessary components of the CPG. Strong hyperpolarization of both C2s or of 3 DSIs blocks motor pattern generation (Getting and Dekin 1985). Furthermore,
although the swim motor pattern could be produced with one C2 hyperpolarized, interfering with G-protein signaling in a single C2 is sufficient to halt the motor pattern (Clemens and Katz 2003). However, we found that changing the spiking activity of these neurons within the range tested did not affect the periodicity of the motor pattern. These results suggest that DSI and C2 each have a permissive, gating effect on the oscillator. That is, their spiking activity is necessary to allow the motor pattern to proceed, but their individual membrane potentials and temporal spiking patterns are not directly involved in generating the oscillations.

A permissive rather than rhythmic role is supported by the observation that the motor pattern can be elicited by tonic firing in either DSI or C2. When a single DSI is made to fire at a constant frequency, the other DSIs are often silent (see Fig. 4C), presumably because of recruitment of I-cell inhibition (Getting and Dekin 1985). Thus it is unlikely that rhythmic activity arises as a consequence of the bursting in other DSIs. It is possible that the tonic firing in C2 and DSI is sculpted by rhythmic presynaptic inhibition. That is, although the neurons fire tonically, they could be releasing neurotransmitter rhythmically onto other CPG neurons. An underlying membrane potential oscillation, which could serve to gate synaptic output, was often observed during periods of tonic spiking (see Fig. 8B). Such a translation of tonic activity into phasic bursting has been reported in the lobster stomatogastric system (Coleman and Nusbaum 1994).

A permissive role for DSI activity in rhythmic motor pattern production is further supported by the behavior of the DSIs after treatment with the serotonin precursor, 5-hydroxytryptophan (5-HTP). 5-HTP treatment increases the serotonin content of the DSIs and potentiates the actions of these serotonergic neurons (Fickbohm and Katz 2000). However, after 5-HTP treatment, the DSIs drop out of the nerve-evoked motor pattern after initially bursting, perhaps because of enhanced recruitment of inhibition, whereas other neurons continue to burst. This suggests that serotonin (5-HT) released from the DSIs is sufficient to elicit the motor pattern and that bursting may be merely a mechanism for delivering enough 5-HT to the oscillator; bath application of serotonin produces a swim motor program with a periodicity similar to that of the swim evoked by stimulation of afferent nerves (McClellan et al. 1994).

**Temperature sensitivity**

After determining that the activity levels of CPG neurons did not affect the periodicity of the motor pattern, we looked for other factors that might play a role. No neuromodulatory substances have been found to affect the period of the motor pattern (McClellan et al. 1994). We observed that the period of the fictive motor pattern was strongly dependent on temperature; there was a 3-fold decrease in period with a 10°C increase in temperature. The cycle period of the motor pattern produced by the isolated nervous system exhibited a temperature sensitivity similar to that of the animal’s behavior, suggesting that the sensitivity of the behavior to changes in temperature are attributed to mechanisms in the CNS rather than neuromuscular or sensory responsiveness. Furthermore, the temperature sensitivity was apparent even when the motor pattern was elicited from within the circuit (by stimulating C2), suggesting a temperature-sensitive mechanism intrinsic to the CPG, rather than a temperature-sensitive afferent input.

Although temperature altered the cycle period, the phase relationships of the CPG neurons did not change with temperature. This suggests that the mechanisms underlying the production of the rhythmic activity are likely to be the same at the different temperatures and that the cause of the change in period is not attributed to a shift to a different mode of oscillation. Thus the CPG is capable of producing a wide range of periods, although this periodicity is not determined by the electrical activity of the CPG neurons.

The periodicities of many rhythmic behaviors, particularly those produced by poikilotherms, show a strong dependency on temperature. Examples are widespread and diverse and include *Aplysia brasiliana* swimming (von der Porten et al. 1982), locust flight (Xu and Robertson 1994), electric fish communication signals (Dunlap et al. 2000), and cricket and cicada calling songs (Fonseca and Revez 2002; Pires and Hoy 1992). The oscillation frequency of individual bursting pacemaker neurons also shows temperature sensitivity resulting from the temperature sensitivity of ion channels (Johnson et al. 1992; Thompson et al. 1986; Treistman and Bablanian 1985). Many neuronal processes, such as synaptic transmission, action potential duration, and enzyme activity, are temperature dependent (Hakozaki et al. 1989; Janssen 1992; Johnson et al. 1991; Joyner 1981; Montgomery and Macdonald 1990; Thompson et al. 1986). Thus the fact that the cycle frequency of the *Tritonia* swim motor pattern is temperature sensitive does not help elucidate the mechanisms underlying frequency regulation in this system; it merely establishes that the system is capable of oscillating at different periodicities.

**Where is the oscillator?**

Our results appear enigmatic because the CPG neurons are all necessary for the motor pattern, yet none is capable of altering the periodicity of the pattern by itself. It is possible that there are important neurons missing from the known CPG circuit. For example, the unidentified I-cells whose inhibitory PSPs are recruited by the DSIs (Getting and Dekin 1985) could be a part of the network oscillator.

An alternate hypothesis is that the oscillations are based on the dynamics of second-messenger signaling. Getting’s simulation of the circuit suggested an important role for the time courses of slow synaptic potentials in setting the periodicity of the motor pattern (Getting 1989a,b). Recent work demonstrates that changes in the time course of synaptic potentials in a CPG can have a greater effect on cycle period than changes in their amplitude (Prinz et al. 2003).

Slow synaptic potentials in the *Tritonia* CPG are likely to be mediated by second-messenger signaling (Clemens and Katz 2001) and G-protein signaling is necessary for the production of the motor pattern (Clemens and Katz 2003). Thus the reason that the oscillator is insensitive to the resting potential level could be that it is a biochemical oscillator (Goldbeter 1996) rather than a voltage-controlled oscillator (Benson and Adams 1987). Calcium oscillations can arise through an interaction of intracellular calcium stores with membrane Ca2+ channels (Friel 1995). Similarly, it has been proposed that cAMP levels can oscillate.
Other rhythmic behaviors that have been hypothesized to be produced by network oscillators include leech swimming (Brodffuehrer and Thorogood 2001), Cline swimming (Arshavsky et al. 1998), Xenopus embryo swimming (Dale and Kuenzi 1997), lamprey swimming (Arshavsky et al. 1993), gastropod feeding (Elliott and Susswein 2002), the crustacean gastric mill movements (Manor et al. 1999), and mammalian respiration (Del Negro et al. 2002). These networks differ in the number of neurons involved and in their complexity. Some are too complex to test the roles of spiking activity in individual CPG neurons. The Tritonia swim CPG is of intermediate complexity; it is more complex than a half-center oscillator, but still has a manageable number of neurons. The insights into the control of rhythmic pattern generation gained by the study of this model system are likely to be applicable to other network oscillators.

ACKNOWLEDGMENTS

We are grateful to W. Frost for assistance and insightful input. We thank S. Pallas, J. Newcomb, E. Hill, and N. Darghouth for important comments on this manuscript.

Present address of S. Clemens: Emory University School of Medicine, Department of Physiology, 615 Michael St., Atlanta, GA 30322 (E-mail: sclemens@emory.edu).

GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-35731 to P.S. Katz. D. Davis was supported by a McNair Scholarship.

REFERENCES


