The Journal of Experimental Biology 206, 105-115

doi:10.1242/jeb.00050

Tension sensitivity of the heart pacemaker neurons in the isopod crustacean *Ligia pallasii*

Akira Sakurai*,† and Jerrel L. Wilkens

Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

*Author for correspondence (e-mail: akira@gsu.edu)

†Present address: Department of Biology, Georgia State University, Atlanta, GA 30303, USA

Accepted 23 September 2002

Summary

In the crustacean neurogenic heart, the cardiac ganglion (CG) acts as a peripherally located central pattern generator (CPG) by producing rhythmic motor output that initiates the heartbeat. In the isopod *Ligia*, the CG consists of six electrically coupled neurons that all function both as endogenous oscillators and as glutamatergic motoneurons innervating heart muscle. In the present study, we present several lines of evidence to suggest that the CG neurons are sensitive to passive stretch and active tension of the heart muscle. Stretching the heart wall caused a sustained decrease in the burst frequency of the CG neuron. Releasing from the stretch caused a rebound increase in burst frequency above the control rate. A brief stretch (200–300 ms duration) caused either phase advance or phase delay of the following CG bursts, depending on the timing at which the stretch was applied. Repeated brief stretches could entrain the CG bursts to either higher or lower frequencies than the free-run burst frequency. Intracellular recording from one of the CG neurons revealed that it exhibited hyperpolarization during the stretch. The stretch-induced hyperpolarization was followed by a burst discharge upon release from the stretch. With increased stretch amplitude, the amplitude of hyperpolarizing response increased and the timing of the following burst was advanced. When the myogenic activity of the heart muscle was pharmacologically isolated from the ganglionic drive by applying a glutamatergic antagonist, Joro spider toxin (JSTX), the spontaneous muscle contraction caused a hyperpolarizing deflection in the CG neuron. Under specific conditions made by JSTX and tetrodotoxin, the CG burst became entrained to the myogenic rhythm. These results suggest that the *Ligia* CG neurons have tension sensitivity in addition to their pacemaker and motoneuronal functions. Such multifunctional neurons may form a single neuron reflex arc inside the heart.

Key words: heart pacemaker, cardiac ganglion, proprioceptive feedback, single neuron reflex, stretch sensitivity, tension sensitivity, crustacean, isopod, *Ligia pallasii*.

Introduction

The crustacean cardiac ganglion (CG) can be classified as one of the simplest forms of motor pattern generators (Cooke, 1988, 2002). The CG consists of a small number of neurons and is located on the heart wall. The CG is capable of producing an endogenous burst discharge without any extrinsic cues, and thus it functions as the primary pacemaker for the heartbeat (reviewed by Maynard, 1960; Prosser, 1973). Periodic bursts of impulses generated in the CG motor neurons produce excitatory junctional potentials (EJPs) in the heart muscle and cause its rhythmic contraction (Irisawa et al., 1962; Brown, 1964; Van der Kloot, 1970; Anderson and Cooke, 1971; Kuramoto and Kuwasawa, 1980). As a simple nerve-effector organ that is capable of functioning independently from the central nervous system, crustacean hearts provide very useful model systems for studying neural mechanisms underlying endogenous rhythmicity, integration of extrinsic inputs, neuromuscular transmission and neurohumoral modulation (reviewed by Maynard, 1960; Prosser, 1973; Cooke, 1988, 2002).

In a variety of rhythmic motor systems, proprioceptive feedback plays important roles, such as changing the amplitude and the frequency of ongoing movement and cycle-by-cycle correction of rhythmic behaviors (Rossignol et al., 1988). Proprioceptive feedback is generally formed by afferent mechanosensory neurons, or proprioceptors, that are found even in peripherally located central pattern generators (CPGs) such as the crustacean stomatogastric nervous system (Simmers and Moulins, 1988; Katz et al., 1989). In crustacean hearts, it has long been proposed that the pacemaker activity of the CG neuron is strongly affected by rhythmic ventricular movement through an unknown feedback system within the heart (Carlson, 1906; Maynard, 1960; Holley and Delaleu, 1972; Kuramoto and Ebara, 1984, 1985; Cooke, 1988, 2002). As there are no sensory neurons in the heart, it has been assumed that the CG neurons themselves possess mechanosensitivity. Alexandrowicz (1932) reported that dendrite processes of the CG neurons near the ganglion trunk

differ from their major peripheral processes and that they are sensitive to mechanical stimulation. He suggested that these fine dendrites mediate the stretch-induced responses of the CG neurons. Since then, several investigators have attempted to show the direct effects of continuous stretch or inflation of the heart on the heartbeat rhythm (e.g. Maynard, 1960; Kuramoto and Ebara, 1984, 1985; Wilkens, 1993). However, direct evidence for the cycle-by-cycle feedback from the heart muscle to the neurons has not been presented. In the present study, we investigated the effects of passive stretch and active muscle contraction on the bursting activity of CG neurons by using opened heart preparations obtained from the isopod crustacean *Ligia pallasii*.

The *Ligia* tubular heart is composed of a single layer of striated muscle fibers aligned in a right-hand spiral (Fig. 1A; Alexandrowicz, 1953; Yamagishi and Ebara, 1985). Unlike decapod hearts, the heart muscle possesses myogenicity that is usually hidden by the neurogenic drive from the CG (Yamagishi and Hirose, 1997; Yamagishi et al., 1998). The *Ligia* CG is composed of six neurons that lie longitudinally along the midline of the inner dorsal wall (Fig. 1B). All of the six CG neurons are endogenous bursters and they fire synchronously due to strong electrical coupling between them (Yamagishi and Ebara, 1985). In addition to the pacemaker function, they also function as the glutamatergic motoneurons innervating the heart muscle (Sakurai et al., 1998) and they exhibit both spike-mediated and graded neuromuscular transmission to the heart muscle (Sakurai and Yamagishi, 2000). Taking advantage of such simplicity, together with knowledge of the *Ligia* heart obtained from previous studies, we report here that contraction of the heart muscle affects the bursting activity of CG neurons by directly affecting their membrane potential. Some of the present results were previously published in an abstract form (Sakurai and Wilkens, 2000).

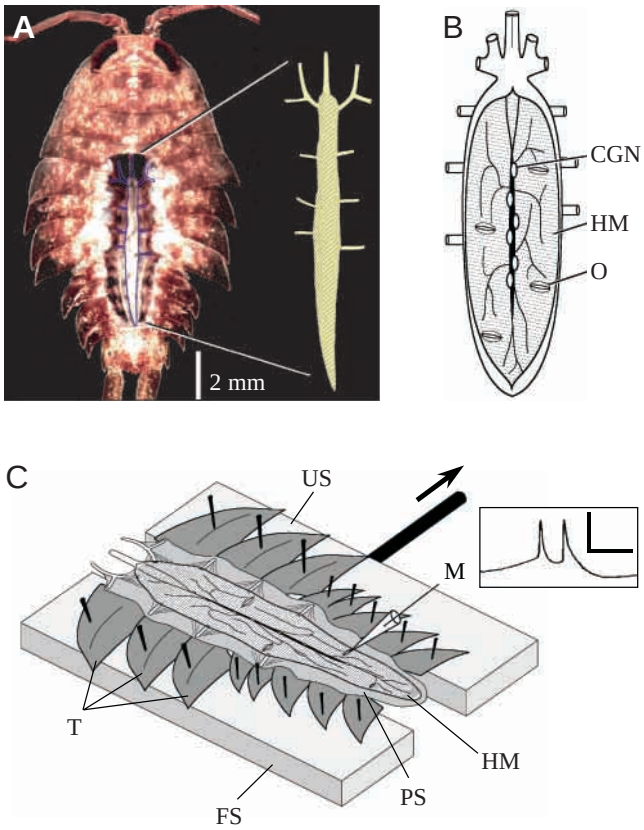

Fig. 1. The *Ligia* heart and the experimental setup. (A) Dorsal view of *Ligia exotica* showing the location of the tubular heart. The dorsal carapaces were partly removed to view the heart. (B) A schematic drawing of an opened heart viewed from the ventral side. CGN, cardiac ganglion neuron; HM, heart muscle; O, ostium. (C) A schematic drawing of the experimental setup for transversely stretching an opened heart. FS, fixed stage; HM, heart muscle; M, microelectrode; PS, pericardial septum; T, tergite; US, unfixed stage. After dissection (see Materials and methods), the lateral rims of the tergites remained attached to the heart *via* pericardial septum. The tergites were pinned on two separate Sylgard stages. One stage was pinned onto the bottom of the experimental chamber and the other stage was connected to a servomotor *via* a steel wire. By displacing the unfixed stage (arrow), one can apply stretch to the heart muscle. The ganglionic bursting activity was monitored by recording the CG-evoked excitatory junctional potentials (EJPs) intracellularly from the heart muscle. The inset shows a single burst of two EJPs recorded from the ostial muscle (calibration: 20 mV, 0.1 s).

Materials and methods

Animals

Adult males and females of the littoral isopod *Ligia pallasii* Brandt, 15–32 mm in body length, were used. They were collected at Pacific seashores of Bamfield Marine Station, Vancouver Island, Canada and were kept in the laboratory at room temperature (18–24°C). More than 100 animals were used in this study.

Heart preparations

Isolated heart preparations were made as described elsewhere (Sakurai et al., 1998; Sakurai and Yamagishi, 2000). After decapitation, the animal was pinned ventral side up in the experimental chamber and the heart was exposed by removing the ventral carapace, ventral nerve cord and visceral organs. At this point, the maximum and minimum values of the diameter of a beating tubular heart were measured at the seventh body segment under the microscope to calculate changes in the circumference. The calculated values were later used to determine the physiological range of the width changes imposed on the opened heart by stretches (Fig. 2B). Next, the heart was opened by longitudinal incision of its ventral wall. The preparation was then pinned dorsal side up and the heart was exposed from the dorsal side along the midline. The suspensory ligaments were all removed except for the pericardial septum. After these treatments, the opened heart became a membranous muscular sheet suspended from the remained lateral rims of the tergites by pericardial septum. The preparation was then laid across two Sylgard stages with the

ventral side up. The tergites were all pinned firmly onto the stages (Fig. 1C). One stage was fixed onto the bottom of the chamber and the other was connected to a servomotor (Model 300 Ergometer, Cambridge Technology, Cambridge, MA, USA) with a steel wire. For intracellular recordings from the CG neuron, the posterior region of the ganglionic trunk was desheathed to expose the somata. Then, the posterior half of the heart wall was pinned extensively onto the fixed stage to prevent movement by stretching (Fig. 5A). After these procedures, the preparation was left for at least 2 h in running saline to restore regular heartbeat.

Stretching the heart wall

By moving the unfixed stage, the opened heart could be either stretched or slackened. Direction of the stretch was carefully adjusted to be in parallel with the muscle fibers. The control position of the unfixed stage was adjusted to set the width of the opened heart to be the median value of the calculated circumferential changes. At the control position, the heart muscle was under moderate tension. To produce brief stretches and sinusoidal stretches, square pulses and sinusoidal waveforms were generated by a conventional function generator; they were sent to the servomotor input. The square pulses were smoothed by a hand-made RC circuit to prevent abrupt stretches that would cause severe damage to the heart and surrounding connective tissues. In most recordings, the magnitude of the applied stretch was shown by the command output from the RC circuit, which was calibrated later as the distance of the displacement of the unfixed stage. In some experiments the changes in the opened heart width was directly measured under the microscope during the experiments, as the actual dimension change was slightly less than the amplitude of the displacement due to elasticity of connective tissues (Fig. 2B).

Solutions

Throughout the experiments, the experimental chamber was continuously perfused with aerated physiological saline of the following composition: NaCl 577 mmol l^{-1}, KCl 14 mmol l^{-1}, $CaCl_2$ 25 mmol l^{-1}, $MgCl_2$ 21 mmol l^{-1}, Na_2SO_4 4.5 mmol l^{-1} and Tris 5 mmol l^{-1} (Yamagishi and Ebara, 1985). The pH was adjusted to 7.4–7.6 using HCl. In some experiments, tetrodotoxin (TTX; Sigma), Joro spider toxin (JSTX; Sigma) and picrotoxin (Sigma) were added to the saline. They were made up in saline just before use.

Electrophysiology

Conventional glass capillary microelectrodes filled with 3 mol l^{-1} KCl (resistance, 15–30 MΩ) were used for recording intracellular electrical activity of the heart muscle and the CG neuron. The tension of the heart muscle was recorded by connecting the unfixed stage to a Pixie force transducer (developed by R. K. Josephson and D. Donaldson; reported by Miller, 1979). Although we attempted to measure the isometric tension generated by the heart, the muscle still shortened a small amount due to elasticity of the muscle itself and the surrounding connective tissues. The electrical signals were sent to a personal computer *via* an A/D converter with commercial data acquisition software (Experimenter's WorkBench, DataWave, Longmont, CO, USA).

Results

Effects of stretching the heart wall on ganglionic bursting activity

To determine whether the heartbeat rhythm is affected by stretching the heart wall, we first applied long steady stretches of various amplitudes to the opened heart to see their effects on the ganglionic bursting activity (Fig. 2). The stretch caused a sustained decrease in the frequency of the CG bursts. Fig. 2A shows a representative series of recordings, in which the bursting activity of CG neurons was monitored by recording the excitatory junctional potentials (EJPs) from the heart muscle while applying 10 s stretches. In the figure, EJPs appear as periodic bursts of rapid depolarizing deflections surmounted on a slow membrane potential wave in the heart muscle (see Fig. 1C inset). There was an apparent drop in the EJP burst frequency at the onset of the stretch, and then the frequency partially recovered and stayed at relatively constant level for the rest of the stretch. Immediately after release from the stretch, the burst frequency increased to more than the free-run rate and then returned to the control level within a few bursts.

The EJP burst frequency decreased with increased stretch amplitude; however, the EJP burst frequencies showed a nonlinear relationship with the dimension changes of the heart (Fig. 2B). Stretches that changed the opened heart width within the physiological dimension range (shown by a horizontal bar in the graph) had little effect on the ganglionic burst frequency. The stretch-induced inhibition became apparent when the heart was stretched more than the estimated diastolic dimension.

Brief stretches caused phase shifts in ganglionic bursting activity

To determine whether there is any cycle-by-cycle feedback from the heart muscle to the CG, we examined the effects of brief stretches applied at various times during the inter-burst period (Fig. 3). A brief stretch produced either phase advance or phase delay of the ganglionic burst cycle, depending on the phase of the cycle in which it was presented. Fig. 3A shows representative records showing the effects of brief stretches (200 ms duration, 0.7 mm amplitude) applied at two different phases of the burst cycle. When the stretch was presented immediately after the first EJP, the second EJP in the burst was suppressed and the following burst cycle was advanced (Fig. 3Ai). By contrast, when the stretch was presented at a later phase, the occurrence of the next burst was delayed (Fig. 3Aii). The phase–response curve increased monotonically as the stretch was applied at later phases of the burst cycle (Fig. 3B). The graph shows the occurrence of both phase advance and phase delay, with a null point located between 20% and 50% of the burst interval (N=3).

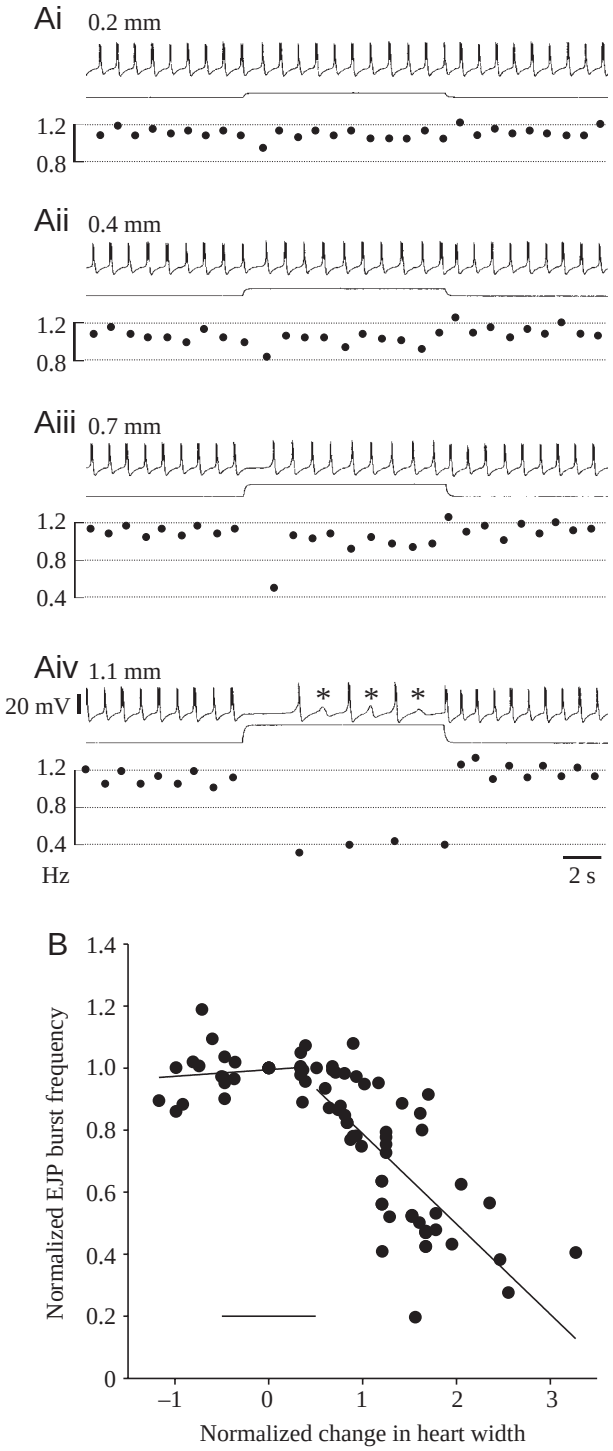

Fig. 2. Effects of long steady stretches on the heartbeat rhythm. (Ai–iv) In each record: top trace, membrane potential recording from the heart muscle showing bursts of excitatory junctional potentials (EJPs); middle trace, displacement of the unfixed stage, showing a 10 s stretch; bottom trace, a plot of instantaneous frequency of the EJP burst. The amplitude of displacement is shown on top of each record. Asterisks indicate slow muscle depolarization without EJPs. The records were obtained from a single preparation. The width of the opened heart during the 1.1 mm stretch (Aiv) was 2.9 mm, which is 38% larger than the calculated diastolic dimension (2.1 mm). The 1.1 mm stretch was approximately 2.5× larger than the amplitude of the circumferential dimension change in a given heart. (B) Steady-state burst frequency during the stretch (duration, 10–30 s) was plotted against amount of extension of the opened heart. Values for the burst frequency were normalized to the free-run frequency, and the change in width of the opened heart was normalized to the estimated circumferential dimension change in a beating heart tube (see Materials and methods). The horizontal bar in the graph shows a range of the changes in opened heart width (±0.5) that is equivalent to the circumferential dimension change in a beating heart. The control position is set at the median value between the maximum and minimum circumferential dimensions. Regression lines were calculated from data with the heart width less than the diastolic dimension (normalized heart width change <0.5, y=0.02x+0.99, r^2=0.03) and from data with the heart width larger than the diastolic dimension (normalized heart width change >0.5, y=–0.29x+1.08, r^2=0.53). Data points were obtained from 11 preparations.

Repetitive brief stretches entrained the ganglionic bursting activity to either higher or lower frequencies than the free-run burst rate. (Fig. 4). In the preparation shown in Fig. 4A, the free-run burst rate was in the range of 1.44–1.61 Hz. Brief stretches (duration, 300 ms; amplitude, 0.7 mm) were applied at (i) 1.0 Hz, (ii) 1.3 Hz, (iii) 1.7 Hz and (iv) 2.0 Hz. When the stretches were applied at frequencies slightly lower (Fig. 4Aii) or higher (Fig. 4Aiii) than the free-run frequency, the EJP bursts became entrained to the applied stretches. The CG bursts could not follow the stretches applied at 1.0 Hz and 2.0 Hz, and the burst rhythm became irregular (Fig. 4Ai,iv). The EJP bursts had one-to-one relationships with the brief stretches from 1.1 Hz to 1.8 Hz (Fig. 4B).

Throughout the experiments, we often noticed that the brief stretches also caused hyperpolarizing deflections in the heart muscle (e.g. Fig. 3Aii). This phenomenon probably resulted from a sudden cessation of tonic transmitter release from the CG neurons (cf. Sakurai and Yamagishi, 2000), which was induced by a stretch-induced hyperpolarization (see below). However, we cannot exclude the possibility that the heart muscle may also respond to stretch in the same way as do molluscan heart muscles (Irisawa, 1978; Jones, 1983). In this study we made no further investigation into this problem.

Membrane potential responses of CG neurons to passive stretch

We next examined the membrane potential response of the CG neuron to the stretch. As the six CG neurons are all electrically coupled, local signals evoked in one neuron can propagate electrotonically to all the other neurons (Yamagishi and Ebara, 1985; Yamagishi et al., 1989). Taking advantage of this, we applied stretches to the anterior half of the heart while recording the stretch-induced response from one of the CG neurons in the posterior half (Fig. 5A). To observe the responses clearly, preparations with a bursting rate of <0.5 Hz were selected for the experiments. The stretches were applied during the inter-burst period when the membrane potential became flat.

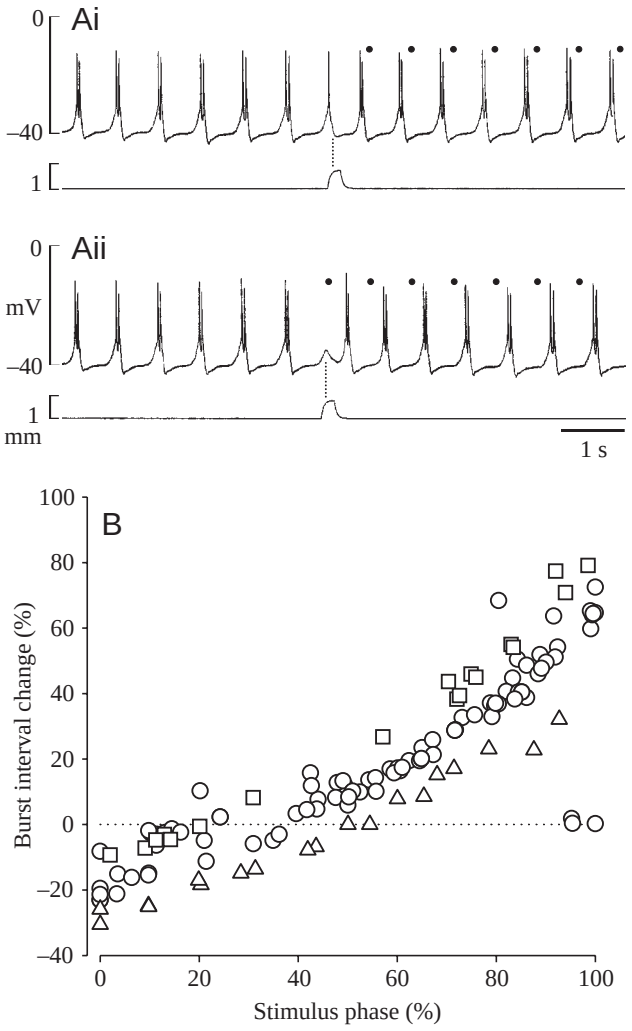

Fig. 3. Effects of brief stretches on the heartbeat rhythm. (A) Example records showing phase advance (i) or phase delay (ii) of the ganglionic burst cycle induced by a brief stretch (duration, 200 ms; amplitude, 0.7 mm). In each record: upper trace, membrane potential of the heart muscle showing rhythmical bursts of excitatory junctional potentials (EJPs); lower trace, applied stretch shown by displacement of the unfixed stage. A brief stretch was given at an earlier phase (i) or at a later phase in the burst cycle (ii). Dots above each record indicate the expected timing of the EJP bursts in the absence of perturbation. The 0.7 mm stretch was approximately 1.4× larger than the estimated amplitude of the circumferential dimension change in a given heart. (B) The relationship between changes in the burst interval and phases of the burst cycle in which the brief stretches were presented. The control burst interval was determined as the average interval of 10 bursts prior to the stretch. Data were obtained from three preparations, and each symbol represents data from one specimen. Free-run burst frequencies were 1.5 Hz (circle), 1.1 Hz (square) and 0.9 Hz (triangle). The stretches were given at a duration of 200 ms (circles and triangles) or 300 ms (squares). The amplitudes of the stretches (circles, 0.7 mm; squares, 0.5 mm; triangles, 0.8 mm) were approximately 1.4× larger than the estimated amplitude of the circumferential dimension change in the given hearts.

Stretching the heart wall produced a hyperpolarizing membrane potential change in the CG neuron (Figs 5, 6). When brief stretch pulses were applied repeatedly at 5 s intervals, the CG neuron exhibited a hyperpolarizing response during each stretch (Fig. 5B). Each hyperpolarizing response was always followed by a rebound burst discharge. As a result, the weakly active CG neuron exhibited rhythmical bursts that were time-locked to the brief stretches. Sinusoidal stretches caused sinusoidal membrane potential changes reflecting the stimulus waveform (Fig. 5C,D). The CG neuron became hyperpolarized at the peak of the sinusoidal stretch and was depolarized and often fired action potentials at the trough of the stretch.

The amplitude of the hyperpolarizing responses increased with increased stretch amplitude (Fig. 6A–C). In normal saline, the hyperpolarizing response became maximum at the beginning of the stretch, and then the membrane slowly depolarized during the stretch (Fig. 6Bi). Relaxation of the heart wall from the control position (downward deflection of the stimulus trace) also induced a burst discharge (Fig. 6Ai) or a slow depolarization (Fig. 6Bi). The rebound burst discharges appeared earlier with increased duration and/or amplitude of the stretches (Fig. 6Ai,D). The hyperpolarizing responses were still observed when neural firing was completely blocked by 1 μmol l^{-1} TTX (Fig. 6Aii). In TTX, the membrane potential stayed at a relatively constant level during the stretch (Fig. 6Bii), and no depolarizing response was induced upon relaxation. In TTX, the hyperpolarizing responses were evoked only when the stretch amplitude became more than 0.2 mm (Fig. 6C). Similar results were obtained from all four preparations examined.

In one preparation, we examined the effect of Ca^{2+}-free saline and picrotoxin (1 mmol l^{-1}), both of which block inhibitory synaptic input from the extrinsic cardioinhibitory neurons (H. Yamagishi, personal communication), on the stretch-induced hyperpolarization. The stretch-induced response was little affected by either treatment, suggesting that the hyperpolarizing response was not mediated by local activation of the inhibitory neurons or any other unknown inhibitory synapses.

Effects of heart muscle contraction on membrane potential activity of the CG neurons

The preceding experiments have demonstrated the effects of artificially applied stretches to the heart wall. However, it was still unclear whether the increased tension or the increased dimension of heart muscle caused the hyperpolarizing responses of CG neurons. To solve this problem, we next attempted to determine the relationship between the active muscle tension and the membrane potential of the CG neuron.

In the *Ligia* heart, we could not produce measurable muscle tension by directly injecting a depolarizing current into a muscle fiber due to very low input resistance. Instead, we took advantage of the myogenicity of the heart muscle. The myogenicity can be pharmacologically isolated from the

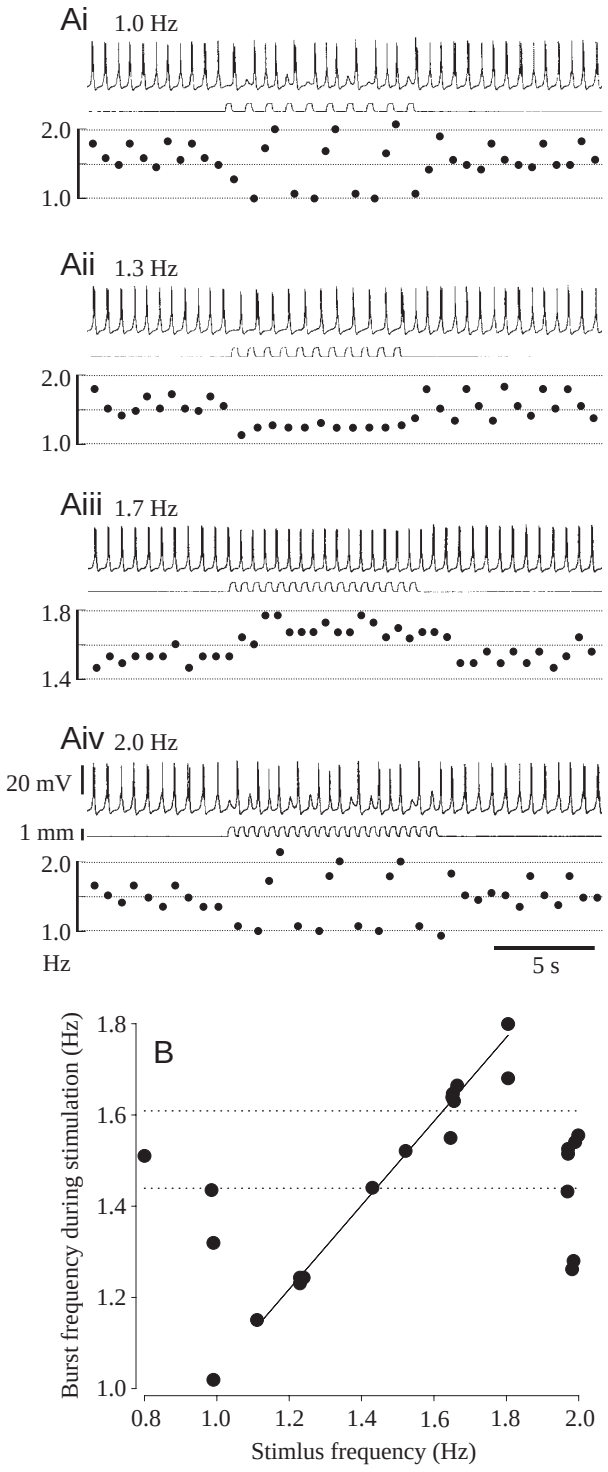

Fig. 4. Effects of repeated brief stretches on the heartbeat rhythm. (A) Example records showing entrainment of the cardiac ganglion (CG) burst rhythm by repeated brief stretches. In each record: upper trace, membrane potential of the heart muscle showing bursts of excitatory junctional potentials (EJPs); lower trace, applied stretch shown by displacement of the unfixed stage. Brief stretches (duration, 300 ms; amplitude, 0.7 mm) were applied at 1.0 Hz (i), 1.3 Hz (ii), 1.7 Hz (iii) and 2.0 Hz (iv). (B) The relationship between the frequency of the CG burst during repeated stretches and the frequency of the stretches. Dotted lines indicate the frequency range of free-running heartbeat (1.44 Hz and 1.61 Hz). A linear regression line in the stimulus range between 1.1 Hz and 1.8 Hz is also shown (y=0.92x+0.11, r^2=0.973). Data shown were obtained repeatedly from a single preparation.

ganglionic drive by blocking the CG-evoked EJPs in the heart muscle with 10 μmol l^{-1} JSTX (Yamagishi et al., 1998; Sakurai and Yamagishi, 2000). Moreover, the myogenic contraction is little affected by tetrodotoxin TTX, which blocks ganglionic bursting activity (Yamagishi and Hirose, 1997). By using JSTX in combination with TTX, we examined the effects of spontaneous muscle contraction on the membrane potential of the CG neuron.

The spontaneous muscle contraction caused a hyperpolarizing membrane potential change in the CG neuron (N=12). Fig. 7 shows representative recordings of the membrane potential activity of the CG neuron and the isometric tension of the heart muscle obtained successively from a single preparation. In this preparation, the CG neuron exhibited regular bursting activity (burst frequency, 0.84–0.88 Hz) in the normal saline. Each CG burst caused a twitch of the heart muscle, showing a transient increase in tension (Fig. 7A). After application of 10 μmol l^{-1} JSTX, the periodic contraction of the heart muscle was temporary abolished, but the CG neuron still fired regularly at a slightly lower frequency (0.79–0.82 Hz) than the control frequency (Fig. 7B). Approximately 5 min after the application of JSTX, the heart muscle started to exhibit myogenic contraction independently from the CG burst (Fig. 7C). At this moment, the contraction occurred in several loci in the heart wall at different rhythms. Under such conditions, the CG burst became irregular and the pacemaker potential of the CG neuron appeared to be interrupted by periodic hyperpolarizing deflections. The hyperpolarizing deflections in the CG neuron corresponded with the periodic increases in tension of the heart muscle (indicated by lines in Fig. 7C). The hyperpolarizing deflections became more apparent when the CG neuron depolarized.

When the CG burst was abolished by the addition of 1.0 μmol l^{-1} TTX, the hyperpolarizing deflections in the CG neuron were seen more clearly corresponding to each muscle contraction (Fig. 7D). Following washout of TTX, the recovered burst discharges tended to occur shortly after each hyperpolarization, as if they were evoked by post-inhibitory rebound (Fig. 7E). In this condition, the bursting activity of CG neurons appeared to be entrained by the myogenic activity of the heart muscle. Thus, the spontaneous muscle contraction had similar effects to the brief stretch, both causing an initial hyperpolarization that was immediately followed by a burst discharge in the CG neuron. This relationship lasted for approximately 40 min after washout of JSTX until the muscle became driven again by the CG burst (not shown). These results strongly support the idea that muscle contraction during the heartbeat affects the pacemaker activity of the CG neurons.

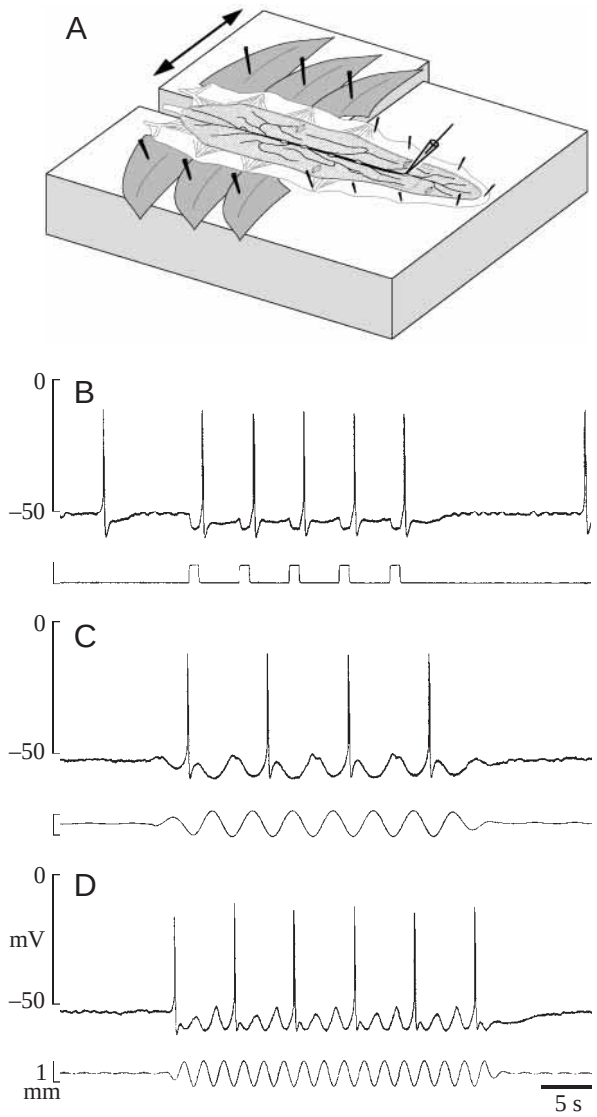

Fig. 5. Membrane potential responses of the cardiac ganglion (CG) neuron to stretches. (A) A schematic drawing of the experimental set-up for applying transverse stretches to the anterior half of the opened heart. After dissection, the lateral rims of only the thoracic tergites remained attached to the heart *via* pericardial septum. The heart wall in the posterior half was pinned extensively onto the fixed stage. (B–D) Membrane potential recordings from the CG neuron. In each record: upper trace, intracellular record from the cell body of the 6th CG neuron (Cell-6); lower trace, applied stretches shown by the amount of displacement of the unfixed stage. In B, stretch pulses (duration, 1.0 s; amplitude, 0.8 mm) were applied repetitively at 0.2 Hz. In C and D, the unfixed stage was moved in sinusoidal waveforms at 0.2 Hz (C) and 0.5 Hz (D).

Discussion

In this study, we first demonstrated that ganglionic bursting activity was suppressed when the heart wall was stretched. By contrast, release from the stretch produced a rebound excitation. These results appeared contradictory to the previous hypothesis for the stretch sensitivity of the CG neurons. The hypothesis for the stretch sensitivity of the CG neurons was originally proposed from the observation that a continuous stretch or inflation of the heart had an excitatory effect on ganglionic pacemaker activity (reviewed by Maynard, 1960; Cooke, 1988, 2002). Kuramoto and Ebara (1984, 1985) showed that inflation of an isolated heart by high perfusion rate caused a gradual increase in the frequency and tonus of the heartbeat in the spiny lobster *Panulirus japonics*. They interpreted this as supporting the idea that stretch has excitatory effects on the heart pacemaker. However, Wilkens (1993) showed that increased perfusion pressure on a semi-isolated heart had a minimal effect on heart rate, or could even slow it, but the perfusion of aerated saline into hypoxic hearts was strongly stimulatory. He concluded that the excitatory effects of high perfusion pressure are largely due to the increased oxygen delivery to the CG. Thus, the stretch sensitivity of the CG neurons has not been directly proved as providing the proprioceptive feedback from the heart muscle to the CG.

In the opened heart preparation made from the isopod tubular heart, the CG is located on a single layer of muscle fibers, and all neural processes are constantly exposed to the freshly perfused saline. Thus, the mechanical stimulation to the heart does not cause changes in delivery of fresh saline. Furthermore, we demonstrated that a brief stretch could phase shift the CG burst cycle (Fig. 3), and that repetitive brief stretches could either accelerate or slow down the ganglionic bursting *via* repetitive phase advances or phase delays (Fig. 4). These results strongly support the idea that mechanical stimulation to the heart wall affects the pacemaker activity of the CG neurons on a cycle-by-cycle basis.

Membrane potential response of the CG neurons to tension of the heart muscle

The CG neuron showed a hyperpolarizing membrane potential change in response to a stretch applied to the heart muscle (Figs 5, 6) and during a spontaneous muscle contraction (Fig. 7). A common event underlying the passive stretch (expansion) and the active contraction (shortening) is the increase in tension of the heart muscle. Therefore, we conclude here that increased muscle tension, no matter how it is produced, causes hyperpolarization in the CG neuron and consequently inhibits the ganglionic pacemaker activity.

In weakly active CG neurons, the stretch and muscle contraction induced rebound excitation upon release from the stretch (Figs 2, 5, 6). The latency of the rebound burst discharge shortened with increased amplitude and duration of the stretches (Fig. 6C). It is likely that the tension-induced hyperpolarization and the rebound bursting are the underlying mechanisms of the stretch-induced inhibition and the following rebound acceleration of the CG burst rate (Fig. 2), phase shifting (Fig. 3) and the entrainment of the CG burst cycle (Fig. 4). The sequences of phase shifting and entrainment of the ganglionic bursting activity are probably similar to those of the inhibitory synaptic inputs onto endogenous burster as described elsewhere (e.g. Maynard, 1961; Pinsker, 1977a,b).

In general, proprioceptors become excited in response to passive stretch or active contraction of the muscles upon which

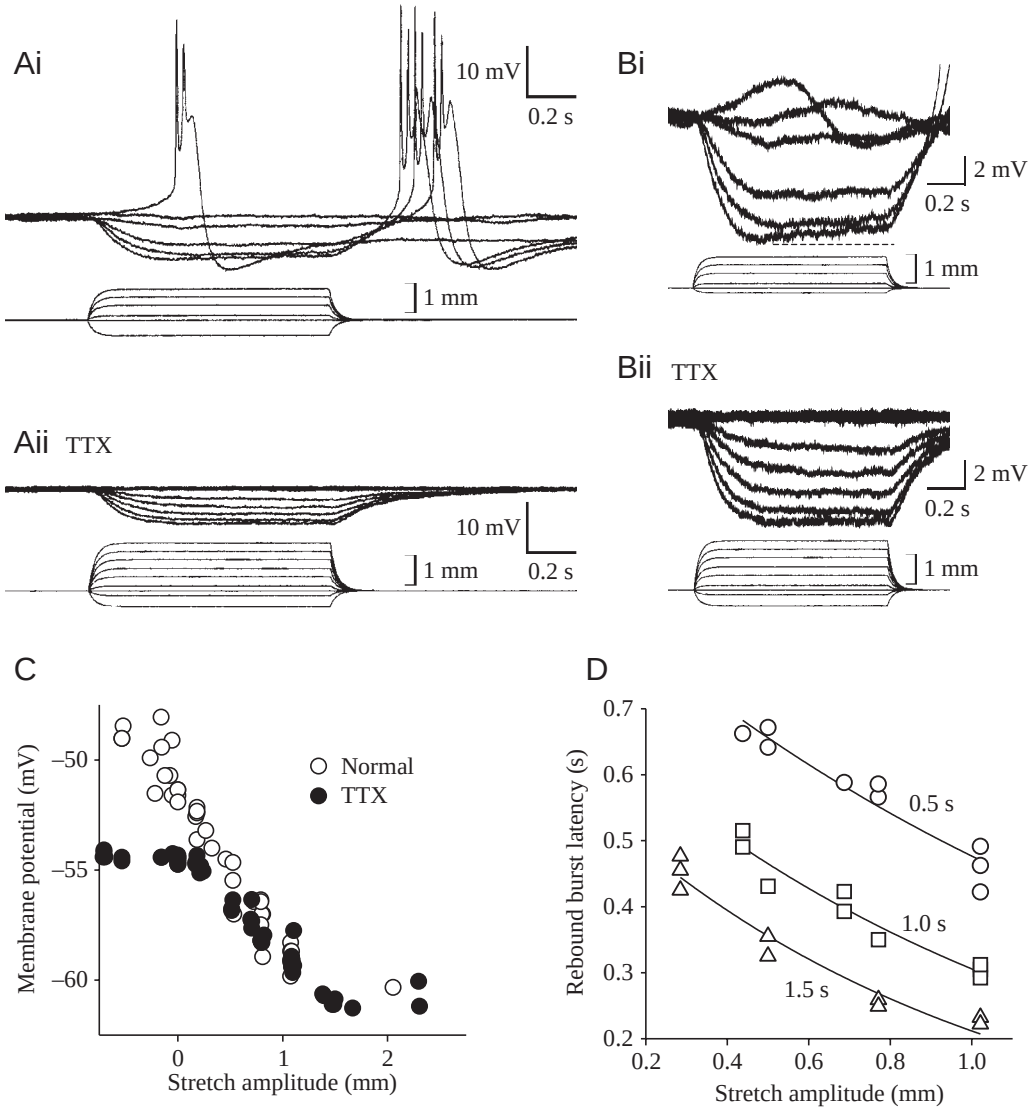

Fig. 6. Membrane potential responses of the cardiac ganglion (CG) neuron to stretches. (A,B) Superimposed traces of intracellular recording from Cell-6 (upper traces) in the normal saline solution (i) and in the presence of 1.0 μmol l^{-1} tetrodotoxin (TTX) (ii). Stretches were applied to the anterior half of the heart and are shown by the amount of displacement of the unfixed stage (lower traces). The records shown here were obtained from a single preparation. In B, the hyperpolarizing responses were shown at higher gain. Note that the stretch-induced hyperpolarization gradually decreased in amplitude during the stretch in the normal saline (Bi) whereas it stayed relatively constant in TTX (Bii). (C) Membrane potential of the CG neuron during the stretch *versus* amplitude of the stretch. Data were obtained repeatedly from a single preparation in the normal saline solution (open circle) and in TTX (filled circle). (D) Time latency for the rebound burst discharge after termination of stretch *versus* amplitude of the stretch. Stretches of various amplitudes were applied with durations of 1.5 s (triangle), 1.0 s (square) and 0.5 s (circle). Data were obtained repeatedly from a single preparation.

their sensory processes project (e.g. Wiersma et al., 1953; Kuffler, 1954; Eyzaguirre and Kuffler, 1955; Eckert, 1961; Eagles, 1978; Proske, 1981; Parsons, 1982; Hartman, 1985). Primary transduction of receptor neurons is generally believed to be linked to the opening of ionic channels (Edwards, 1983). It has been shown in abdominal stretch receptors in crayfish that the stretch-activated channel has low cation selectivity, including sodium, potassium, calcium and magnesium (Nakajima and Onodera, 1969a,b; Brown et al., 1978; Erxleben, 1989; Rydqvist and Purali, 1993). Our data showed that the tension-induced responses of the CG neuron were in the hyperpolarizing direction (Figs 5–7), and the responses became more apparent when the CG neuron was depolarized (Fig. 6C). These results indicate that the tension-induced hyperpolarization was produced by increased ion conductance with the reversal potential located at a more hyperpolarized level than the resting potential. In addition, TTX-sensitive sodium conductance may also be activated directly by tension or indirectly *via* the tension-induced hyperpolarization, as (1) relaxation from a stretch induced a burst discharge or a slow depolarization in the CG neuron, but no such response was induced in TTX (Fig. 6A), and (2) the stretch-induced hyperpolarization gradually decreased in amplitude during the stretch but stayed relatively constant in TTX (Fig. 6B). Such inward current may contribute to the rebound excitation and the phase-advance of the CG burst cycle. In this study, no further attempts were made to identify which ion channels mediate the hyperpolarizing responses of the CG neurons. To characterize the tension-sensitive ion conductances, pharmacological analyses using ion-channel blockers and ion-substitution experiments under voltage clamp are further needed.

At present, it is still unknown where the tension-sensitive sites are located in the processes of the CG neurons. In *Ligia exotica*, fine dendrite processes were observed emerging from the ganglionic trunk region (Sakurai and Yamagishi,

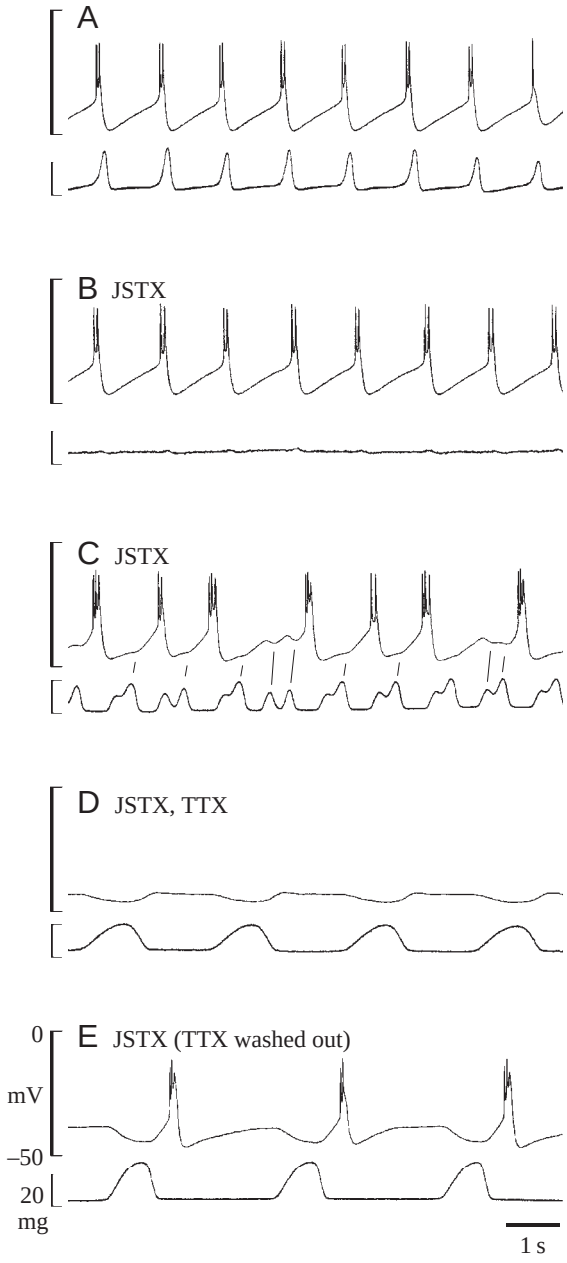

Fig. 7. Effects of spontaneous muscle contraction on the membrane potential of the cardiac ganglion (CG) neuron. In each record: upper trace, the membrane potential activity recorded from Cell-6; lower trace, the myocardial tension recorded from the anterior half of the heart. Records A to E were obtained successively from a single preparation. (A) Control activity of the CG neuron and the heart muscle recorded in the normal saline solution. (B) 3 min after application of 10 μmol l^{-1} Joro spider toxin (JSTX). (C) 7 min after application of JSTX. The JSTX was applied for 17 min, and then the perfusion was switched to the saline containing both JSTX and tetrodotoxin (TTX). (D) 3 min after addition of 1.0 μmol l^{-1} TTX. (E) Bursting activity of the CG neuron recovered 3 min after washout of TTX.

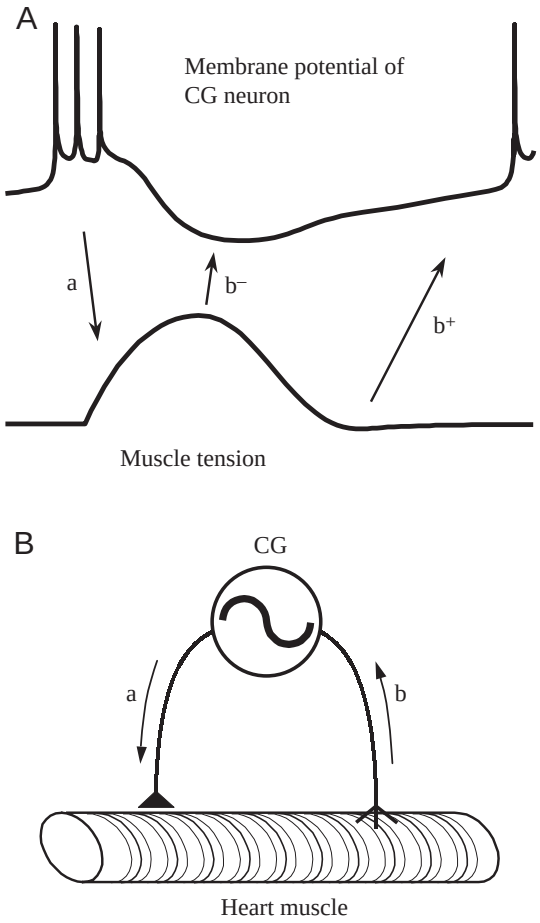

Fig. 8. Interaction between the cardiac ganglion (CG) neurons and the heart muscle. (A) A schematic drawing of the membrane potential activity in the CG neurons and the heart muscle tension. A burst discharge of the CG neurons induces tension of the heart muscle *via* the excitatory neuromuscular transmission (a). The muscle tension has a hyperpolarizing effect on the CG neuron (b^-), which may assist the termination of the burst and enhance the after-burst hyperpolarization in the CG neuron. Relaxation of the heart muscle causes the postinhibitory rebound excitation (b^+), which may advance the following burst discharge. See text for details. (B) A single neuron reflex arc formed by the CG neurons in the *Ligia* heart. The CG neurons function as endogenous oscillators by generating rhythmical burst discharges. They produce rhythmic motor output that is sent to the heart muscle *via* the neuromuscular junctions (a). They are also sensitive to the muscle tension (b), so that the heart muscle activity can entrain the ganglionic bursting activity under specific conditions such as when 'a' is artificially blocked by Joro spider toxin (JSTX).

unpublished observation). These proximal dendrites appeared similar to the hypothetical stretch-sensitive dendrites described in the decapod CG by Alexandrowicz (1932). Detailed anatomy of the CG neurons should further be examined.

Functional significance of stretch sensitivity of the CG neuron

In the open circulatory system of crustaceans, the heart functions as a suction force pump suspended in the pericardial cavity by the elastic ligaments (Maynard, 1960). Tension of the heart muscle becomes maximum during systole, when the

muscles generate the maximum force to overcome the vascular resistance and to extend the suspensory ligaments. By contrast, diastolic expansion is produced by the elastic recoil of the suspensory ligaments. The muscle tension will decline until it equals the recoiling force of the ligaments, which also declines as the heart dilates. Therefore, in an intact beating heart, the tension-induced hyperpolarization in the CG neurons most likely occurs during the systolic contraction, whereas the rebound excitation occurs during diastole.

The actual sequence of events in a beating heart is schematically drawn in Fig. 8A. The CG burst causes a transient increase in the muscle tension *via* excitatory neuromuscular transmission (a). The increased muscle tension then returns the hyperpolarizing feedback to the CG neurons (b^-). This tension-induced hyperpolarization may help terminate ongoing burst and thus may play a role in preventing sustained bursting that will cause a harmful systolic arrest. The tension-induced hyperpolarization may also act as a common inhibitory input to all of the six CG neurons to enhance their synchronized bursting activity (e.g. Kandel et al., 1969; Pinsker, 1977b), although it has been shown previously that the synchronization of the membrane potential activity is produced mainly by the strong electrical coupling (Yamagishi and Ebara, 1985). By contrast, relaxation of the muscle induces a following discharge in the CG neuron (b^+), which can consequently keep the heart rate slightly higher than the free-run burst rate by causing repetitive phase advances in the CG burst cycle. This idea is supported by the fact that the burst frequency of the CG neuron slightly decreased when the muscle contraction was temporary abolished by JSTX (Fig. 7B). Altogether, the tension-induced feedback may contribute to the establishment of a regular rhythm and the optimum motor output for ongoing heartbeat.

There have been a number of studies of extrinsic cardioregulation in crustaceans (Maynard, 1960; Prosser, 1973; Cooke, 2002). In *Ligia exotica*, heart rate and contraction force of the heart muscle are both regulated by the cardioregulatory axons (CI, CA1 and CA2) projected from the central nervous system (Yamagishi et al., 1989; Sakurai and Yamagishi, 1998a,b). Together with the present results, the heart rate and the contraction force are optimized intrinsically *via* the tension-sensitive feedback of the CG neurons themselves (short-range feedback), whereas the total cardiac output is extrinsically controlled from the central nervous system through the extrinsic regulatory neurons and neurohormones (long-range feedback) in response to various behavioral changes and metabolic demands. It will be interesting to determine how the tension sensitivity of the CG neurons is modified by the extrinsic regulatory inputs.

The CG neuron forms a single neuron reflex arc

In the *Ligia* heart, all of the six CG neurons act as the primary pacemaker for the heartbeat by exhibiting endogenous bursting activity (Yamagishi and Ebara, 1985). They also function as the glutamatergic motoneurons innervating the heart muscles (Sakurai et al., 1998). Together with the present results, we conclude here that the CG neurons are multifunctional neurons that possess tension sensitivity in addition to their pacemaker and motor functions.

There have been several examples of the multifunctional neurons that are suggested to form a ‘single neuron reflex arc’ (in the *Hydra* nervous system, Westfall and Kinnamon, 1978; Westfall et al., 1991; in the pharynx of the nematode *Caenorhabditis elegans*, Raizen and Avery, 1994; Avery and Thomas, 1997). The neurosecretory cells possessing a sensory function have also been reported in various animals (in the leech *Hirudo medicinalis*, Wenning et al., 1993; in the lobster *Homarus*, Pasztor and Bush, 1989; in the carotid body of mammals, González et al., 1992; reviewed by Wenning, 1999). A number of sensory neurons are known to function as elements of the pattern generator circuits (e.g. crayfish locomotion, Sillar et al., 1986; locust flight, Pearson et al., 1985; lamprey swimming, Grillner et al., 1991). Having sensory receptors as functional elements of the pattern generator provides the automatic regulation of rhythmical motor output appropriate for the mechanical state of motor organ in various external conditions (Pearson, 1993). To our knowledge, this study is probably the first report that electrophysiologically demonstrated single multifunctional CPG neurons having mechanosensitivity in addition to the endogenous pacemaker function and the motoneuronal function. By having a sensory-motor function without any intermediate synapses, the CG neurons may form the single neuron reflex arc within the heart.

We thank Dr H. Yamagishi for valuable discussions and for kindly providing a working space to A.S. during his stay in Japan. We thank Dr P. S. Katz for comments on the earlier version of this manuscript. We also thank the staff of the Bamfield Marine Station, Bamfield, BC, Canada for hospitality during the collecting trip. Support for this study was provided by a grant from the Natural Sciences and Engineering Research Council of Canada (J.L.W.).

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