

## GRADED NEUROMUSCULAR TRANSMISSION IN THE HEART OF THE ISOPOD CRUSTACEAN *LIGIA EXOTICA*

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### Summary

We present several lines of evidence for the occurrence of graded synaptic transmission in addition to impulse-mediated transmission at the neuromuscular junction between cardiac ganglion (CG) neurones and the myocardium in the isopod crustacean *Ligia exotica*. In the heart of adult *Ligia exotica*, the CG acts as a primary pacemaker for the heartbeat by generating periodic bursts of impulses and entrains the myogenicity of the myocardium *via* impulse-mediated excitatory junctional potentials. When impulse generation was blocked by tetrodotoxin (TTX; 50 nmol l<sup>-1</sup>), the CG neurones and the myocardium periodically exhibited synchronized slow depolarizing potentials. The association between the slow depolarizing potentials in the neurone and the myocardium was eliminated by application of Joro spider toxin (JSTX), a specific glutamate antagonist. When the CG neurone was made quiescent by a higher dose of TTX (1.0 µmol l<sup>-1</sup>), sinusoidal current injected into the CG neurone induced similar sinusoidal membrane potential responses in the myocardium. The sinusoidal muscle responses were eliminated by application of either JSTX or low-Ca<sup>2+</sup>

saline. Under voltage-clamp conditions, the myocardium exhibited periodic inward current responses to sinusoidal current stimuli applied to the CG neurone. The reversal potential for the current response of the myocardium was similar to that of the impulse-mediated excitatory junctional current (EJC). Extracellular macropatch recordings of EJCs made at the neuromuscular junctional site revealed the spontaneous appearance of miniature EJCs asynchronous with the CG spikes in addition to large spike-evoked EJCs. The miniature EJCs were present in saline containing TTX, and their frequency was strongly affected by the slow membrane potential change in the CG neurone. These results suggest that the CG neurones drive the myocardium by graded neuromuscular transmission in addition to impulse-mediated transmission in the heart of *Ligia exotica*.

Key words: graded synaptic transmission, neuromuscular transmission, heart, cardiac ganglion, myocardium, Crustacea, *Ligia exotica*.

### Introduction

Graded synaptic transmission has been demonstrated in many spiking and nonspiking neurones in central and peripheral ganglia (for a review, see Pearson, 1976). In these synapses, the presynaptic neurone releases transmitter as a graded function of presynaptic voltage and produces graded synaptic potentials in the postsynaptic neurone. Graded transmitter release has also been observed at the neuromuscular junction sites on skeletal muscles by manipulating the presynaptic voltage of the nerve terminals (Katz and Miledi, 1965, 1967a,b; Dudel, 1982, 1983; Atwood et al., 1987). However, motoneurones generally produce all-or-nothing impulses that propagate along the peripheral axons to their terminals on muscles. To our knowledge, there have been no reports of any motoneurones that transmit graded motor signals to muscle.

In the heart of many crustaceans, the cardiac ganglion (CG) produces a rhythmic motor pattern that results in the heartbeat

(for reviews, see Maynard, 1960; Prosser, 1973). Periodic bursts of impulses generated in the CG motor neurones produce excitatory junctional potentials (EJPs) in the cardiac muscle and cause myocardial contraction (Irisawa et al., 1962; Brown, 1964; Van der Kloot, 1970; Anderson and Cooke, 1971; Kuramoto and Kuwasawa, 1980). Recently, Yamagishi and Hirose (1997) showed in the isopod *Ligia exotica* that the myocardium also has endogenous automaticity and that the CG acts as a primary pacemaker to entrain the myogenic activity to the neurogenic rhythm *via* impulse-mediated EJPs.

The CG of *Ligia exotica* consists of six spontaneously active neurones, and strong electrical interactions between them cause them to burst synchronously (Yamagishi and Ebara, 1985). In a previous study, we have shown that all six neurones are glutamatergic motoneurones (Sakurai et al., 1998) and that neuromuscular transmission between the CG neurones and the myocardium is blocked by a specific glutamate antagonist, Joro

spider toxin (JSTX) (Sakurai et al., 1998; Yamagishi et al., 1998). In the present study, we further examined neuromuscular transmission between CG neurones and the myocardium in the heart of adult *Ligia exotica* using JSTX in combination with tetrodotoxin (TTX), which blocks impulse generation in the CG. The results suggest that, in addition to impulse-mediated synaptic transmission, graded synaptic transmission occurs at the neuromuscular junction between the CG neurones and the myocardium. Some of these results have appeared in abstract form (Sakurai and Yamagishi, 1997).

### Materials and methods

Adult males and females of the littoral isopod *Ligia exotica* (Roux), 12–20 mm in body length, were used. They were collected from Pacific seashores (Izu and Boso, Japan) and kept in the laboratory at room temperature (22–26 °C). More than 130 specimens were used for the experiments.

The anatomy of the heart has been detailed by Yamagishi and Ebara (1985). The heart is tubular, consisting of a single layer of striated muscle fibres. The CG located on the inner surface of the dorsal heart wall consists of six similarly sized neurones whose somata (diameter 40–60 µm) lie longitudinally along the CG trunk (*Ligia exotica*, Suzuki, 1934; *Ligia oceanica*, Alexandrowicz, 1952). These somata are designated (from the anterior) Cell-1, Cell-2 and so on to Cell-6 (Yamagishi and Ebara, 1985).

Isolated heart preparations were used as described in a previous study (Sakurai et al., 1998). The heart was opened by making a longitudinal incision in the ventral wall to expose the CG and was laid on a bed of petroleum jelly in the experimental chamber with its inner surface upwards. The heart was then gently covered with a nylon mesh to prevent movement. The experimental chamber was perfused with aerated physiological saline of the following composition (in mmol l<sup>-1</sup>): NaCl 577, KCl 14, CaCl<sub>2</sub> 25, MgCl<sub>2</sub> 21, Na<sub>2</sub>SO<sub>4</sub> 4.5 and Tris 5 (Yamagishi and Ebara, 1985). The pH was adjusted to 7.4 using HCl. In some experiments, low-Ca<sup>2+</sup> saline was prepared by replacing CaCl<sub>2</sub> with MgCl<sub>2</sub> to block chemical synaptic transmission. TTX and JSTX were obtained from Wako Pure Chemicals. They were made up in saline just before use. In experiments using TTX, we prepared two types of TTX solution in saline: high-TTX saline (1.0 µmol l<sup>-1</sup>) and low-TTX saline (50 nmol l<sup>-1</sup>).

Conventional glass capillary microelectrodes filled with 3 mol l<sup>-1</sup> KCl (resistance, 15–30 MΩ) were used for recording the intracellular electrical activity of the myocardial fibres and the CG neurones. A glass capillary suction electrode was used for extracellular recording of nerve impulses. For intracellular stimulation of a CG neurone, square-pulse or sinusoidal current was injected into the soma through the recording electrode *via* a bridge circuit. In some experiments, the stimulus electrode was filled with 4 % neurobiotin [*N*-(2-aminoethyl) biotinamide hydrochloride, Vector Labs] solution in 1 mol l<sup>-1</sup> KCl for subsequent visualization of the CG neurones (for details, see Sakurai and Yamagishi, 1998a,b).

The excitatory junctional currents (EJCs) were recorded extracellularly using a macropatch electrode (see Fatt and Katz, 1952; del Castillo and Katz, 1956; Dudel and Kuffler, 1961a,b; Dudel, 1982, 1983; Sakurai et al., 1998). The tip of a microelectrode was broken, bent and heat-polished (inner diameter, 10–20 µm). The electrode was then filled with physiological saline and connected to the current–voltage converter. The resistance of the electrode was 0.5–1 MΩ, and the resistance to ground was 0.8–2 MΩ when the electrode was placed at the final recording position on a myocardial fibre. Neither suction in the electrode nor pressure onto the muscle was applied during the macropatch recording.

To determine the reversal potentials for the membrane current responses of the myocardium, the muscle membrane was held at various potential levels using a two-electrode voltage-clamp technique. Two microelectrodes, one (resistance 10–15 MΩ) for membrane potential recording and the other (resistance 1.5–3 MΩ) for current injection, were inserted into a single myocardial fibre (approximately 30–75 µm wide and 1.5–3.3 mm in length). The distance between the two electrodes was less than 80 µm. There are strong electrical connections among myocardial fibres (Yamagishi and Hirose, 1997). The space constant along a single myocardial fibre was approximately 600 µm and that across the contiguous fibre was approximately 400 µm (Sakurai et al., 1998). Although we could not achieve space-clamp of myocardial fibres, the membrane potential changes caused by current injection deviated by less than 10 % between the sites impaled by the two microelectrodes. When a sinusoidal current stimulus was applied to the CG neurone, the amplitude of the current response was calculated by subtracting the instantaneous stimulus intensity from the recorded current amplitude. The signals were stored on FM tape and displayed on a thermal array chart recorder.

### Results

#### *Correlation of slow membrane potential changes between CG neurones and the myocardium*

Motor control of the myocardium by the CG neurones in the adult neurogenic heart of *Ligia exotica* was examined in opened heart preparations (Figs 1, 2). Fig. 1 shows a representative series of recordings in which the intracellular activity of the CG neurone (top trace), the impulse activity of the ganglionic trunk (middle trace) and the intracellular activity of the myocardial fibre (bottom trace) were recorded simultaneously (Fig. 1A). Since the six CG neurones exhibit spontaneous synchronous bursting activity *via* electrical connections, the membrane potential changes monitored in one neurone represent those occurring in all six neurones (Yamagishi and Ebara, 1985). The impulse activity of the CG neurones evoked EJPs superimposed on the slow depolarizing potentials in the myocardial fibre (Fig. 1B). In semi-intact heart preparations, the EJPs usually induce action potentials in the myocardium whose maximum membrane potential is in the range –60 to –50 mV (see Yamagishi et al., 1989; Yamagishi

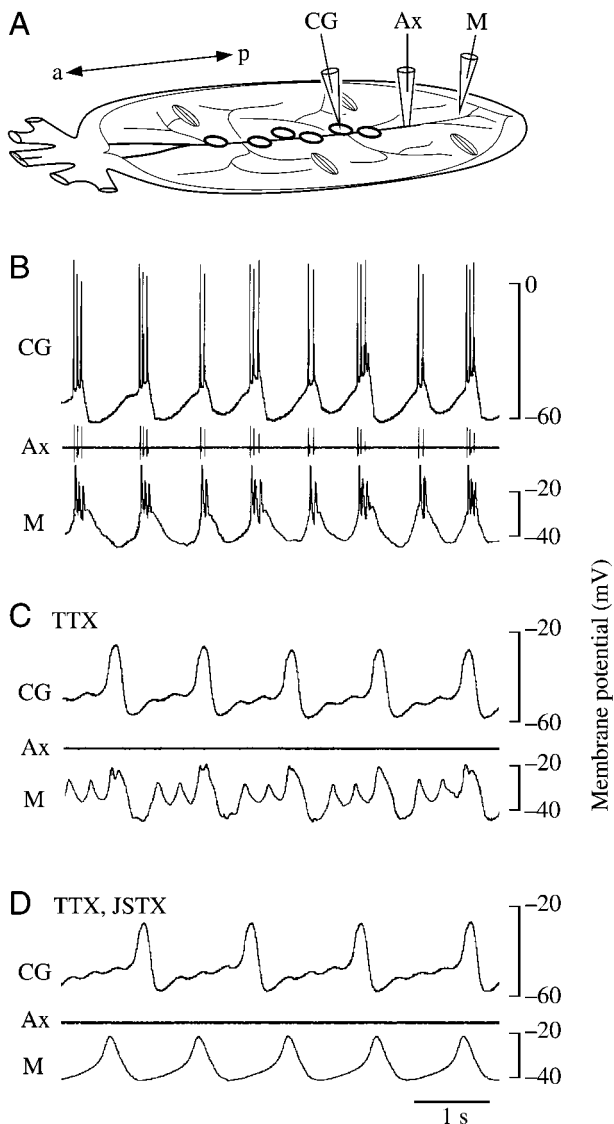


Fig. 1. Effects of tetrodotoxin (TTX) and Joro spider toxin (JSTX) on neuromuscular transmission between a cardiac ganglion (CG) neurone and the myocardium. (A) A schematic drawing of an opened heart preparation. An arrow indicates the anterior (a) and posterior (p) directions. Membrane potential activity of a CG neurone (CG) and the myocardium (M) and impulse activity of CG axons (Ax) were simultaneously recorded in normal saline (control, B), 5 min after the onset of application of  $50 \text{ nmol l}^{-1}$  TTX (C) and 30 min after the further addition of  $10 \mu\text{mol l}^{-1}$  JSTX (D). In each recording, the right-hand voltage scale indicates the absolute value of the membrane potential. Note that membrane potential changes in the myocardium followed those in the CG neurone even when ganglionic impulses were blocked by TTX in C. The two rhythms became independent after the addition of JSTX in D. All recordings were obtained successively from the same preparation.

and Hirose, 1997; Sakurai et al., 1998). However, in the opened heart preparations, the maximum membrane potentials of myocardial fibres were in the range  $-50$  to  $-30 \text{ mV}$ , and the myocardium often failed to generate action potentials, probably because of damage caused by the incision.

In saline containing  $50 \text{ nmol l}^{-1}$  TTX (low-TTX saline), impulse generation in the CG was completely blocked (see Yamagishi, 1998), and the CG neurones and the myocardium both exhibited periodic slow depolarizing potentials (Fig. 1C). Under these conditions, large depolarizing potentials in the myocardium appeared to synchronize with the slow depolarization in the CG neurone. This non-impulse-mediated coupling of the membrane activities between the CG neurone and myocardium was lost after the addition of  $10 \mu\text{mol l}^{-1}$  JSTX (Fig. 1D). In the presence of JSTX, these tissues exhibited independent spontaneous regular rhythms.

We next examined the relationship between the membrane potential changes in a CG neurone and the myocardium by experimentally changing the frequency of neuronal activity (Fig. 2). The results were similar to those shown in Fig. 1: the CG neurone and the myocardium showed synchronized membrane potential changes in both normal and low-TTX saline (Fig. 2A,B). In low-TTX saline, the myocardium exhibited membrane potential oscillations during pacemaker-like depolarization in the CG neurone and following large depolarizing potentials coupled with the depolarization in the CG neurone (Fig. 2B). This coupling was maintained even when the frequency of the neural activity was changed by current injection into the soma. In contrast, current injection ( $\pm 1 \mu\text{A}$ ) into the myocardial fibre had no effect on the CG neurone (not shown). Addition of  $10 \mu\text{mol l}^{-1}$  JSTX abolished this non-impulse-mediated neuromuscular coupling (Fig. 2C).

#### Graded membrane potential responses of the myocardium

The results obtained in low-TTX saline suggest that there is non-impulse-mediated signal transmission from CG neurones to the myocardium in addition to the impulse-mediated EJPs. We next examined the effects of experimentally induced slow membrane potential changes in a CG neurone on myocardial membrane potentials. Fig. 3 shows an example of experiments in which the membrane potential changes in the CG neurones were completely eliminated by saline containing a higher concentration of TTX ( $1.0 \mu\text{mol l}^{-1}$ , high-TTX saline) (see Yamagishi, 1998). In high-TTX saline, the resting potential of the CG neurone was  $-55$  to  $-52 \text{ mV}$ , and the membrane potential of the myocardium exhibited small irregular fluctuations of approximately  $5 \text{ mV}$  in amplitude (also see Figs 4Ai,Bi, 6). Under these conditions, we continuously injected a sinusoidal current ( $\pm 15 \text{ nA}$ ,  $3.2 \text{ Hz}$ ) into the soma of a CG neurone (Cell-6) and recorded the intracellular activity of the myocardium successively from various sites (A–K in Fig. 3) on the heart wall. After the experiments, the neuronal processes of all six CG neurones were stained by intracellular injection of neurobiotin into the soma of the CG neurone through the stimulus electrode (inset drawing in Fig. 3; see Sakurai et al., 1998).

In response to a sinusoidal stimulus to the CG neurone, the myocardium exhibited sinusoidal membrane potential changes (Fig. 3A–K). The amplitude of the muscle response was larger in the posterior half of the heart close to the stimulated neurone (Fig. 3E–H), whereas the myocardial fibres of the anterior

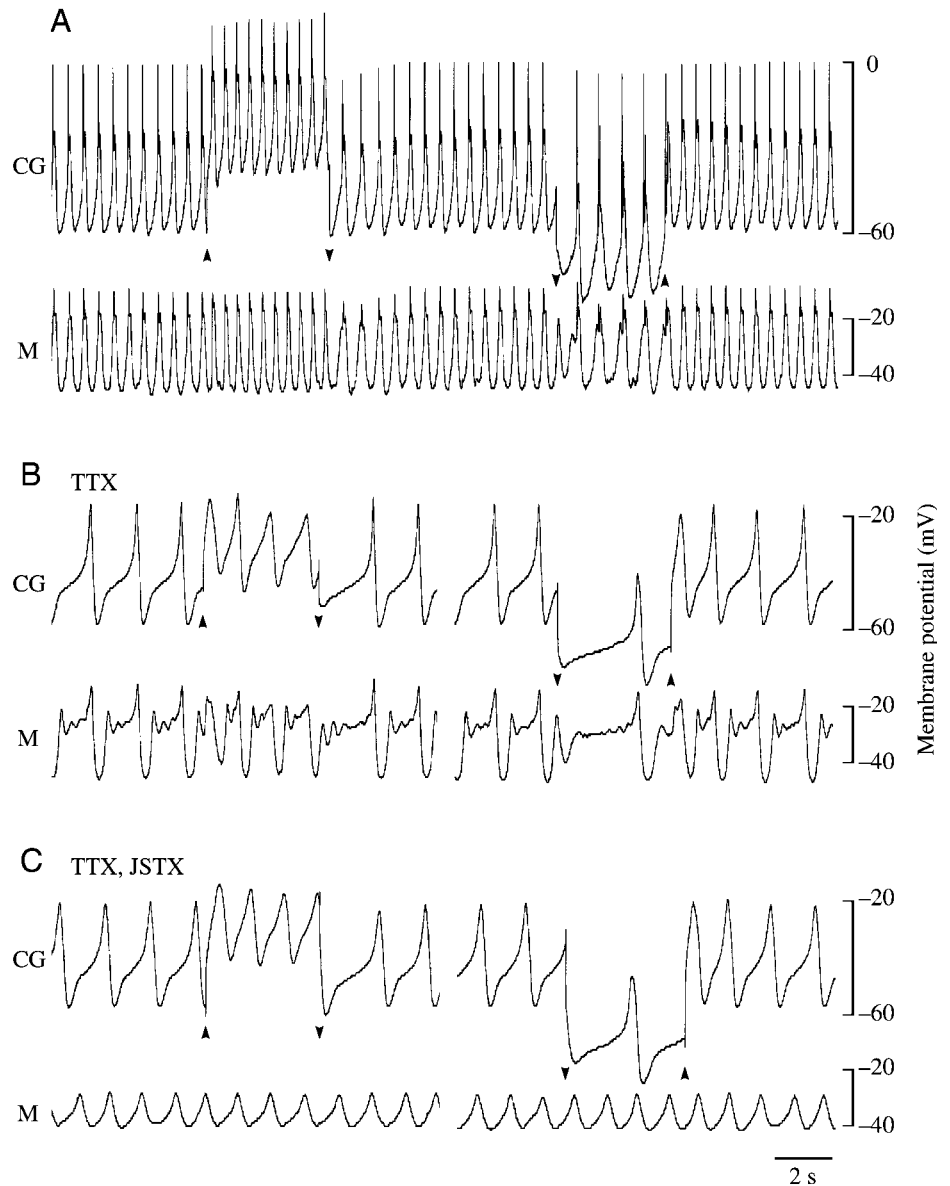


Fig. 2. Effects of current injection into a CG neurone on the membrane potential of a myocardial fibre. The membrane potentials of the CG neurone (CG) and the myocardial fibre (M) were recorded simultaneously in the opened heart preparation. Recordings were obtained before (A), 5 min after the onset of application of  $50 \text{ nmol l}^{-1}$  tetrodotoxin (TTX) (B) and 15 min after the further addition of  $10 \mu\text{mol l}^{-1}$  Joro spider toxin (JSTX) in saline (C). In each recording, the frequency of the membrane potential activity of the CG neurone was changed by injecting steady depolarizing or hyperpolarizing current pulses into the soma of the CG neurone through the recording electrode for the periods shown by arrowheads. The right-hand voltage scales indicate the absolute value of the membrane potential. Note that, both in normal saline (A) and in TTX-containing saline (B), the membrane activities of the neurone and muscle were coupled even when the frequency of the neural activity was experimentally changed. The coupling was lost after addition of  $10 \mu\text{mol l}^{-1}$  JSTX (C). All recordings were obtained successively from the same preparation.

region showed low-amplitude responses (Fig. 3A–C, I–K). A smaller response was also recorded from a region where few axonal processes were stained (Fig. 3D). Similar stimuli applied directly to the myocardium caused only localized responses restricted to within several hundred micrometres of the stimulation site (not shown).

To determine whether the coupling of electrical activities between the CG neurones and the myocardium was induced by chemical or electrical synapses, the effects of JSTX or low- $\text{Ca}^{2+}$  saline on the sinusoidal responses of the myocardium were examined in high-TTX saline. Fig. 4 shows representative results obtained by simultaneous intracellular recording from the soma of a CG neurone (upper trace) and a myocardial fibre approximately  $500 \mu\text{m}$  away from the stimulated soma (lower trace). When the membrane potential of the CG neurone was changed by sinusoidal current injection ( $\pm 15 \text{ nA}$ ) into the soma through the recording electrode, sinusoidal membrane potential responses of approximately

$10 \text{ mV}$  in amplitude were induced in the myocardial fibre (Fig. 4Ai, Bi). These myocardial sinusoidal responses were eliminated after application of either  $10 \mu\text{mol l}^{-1}$  JSTX (Fig. 4Aii) or low- $\text{Ca}^{2+}$  saline (Fig. 4Bii). Addition of JSTX or low- $\text{Ca}^{2+}$  saline also eliminated membrane potential fluctuations in the myocardium and caused a slight hyperpolarization (approximately  $5 \text{ mV}$ ). The sinusoidal response of the myocardium recovered partially after washout of JSTX-containing saline (Fig. 4Aiii) and completely after washout of low- $\text{Ca}^{2+}$  saline (Fig. 4Biii). In both cases, membrane potential fluctuations in the myocardium reappeared after the washout.

#### *Reversal potential for graded membrane current responses of the myocardium*

To determine the reversal potential for the responses of the myocardium to sinusoidal current injection into a CG neurone, the membrane current responses of the myocardium were

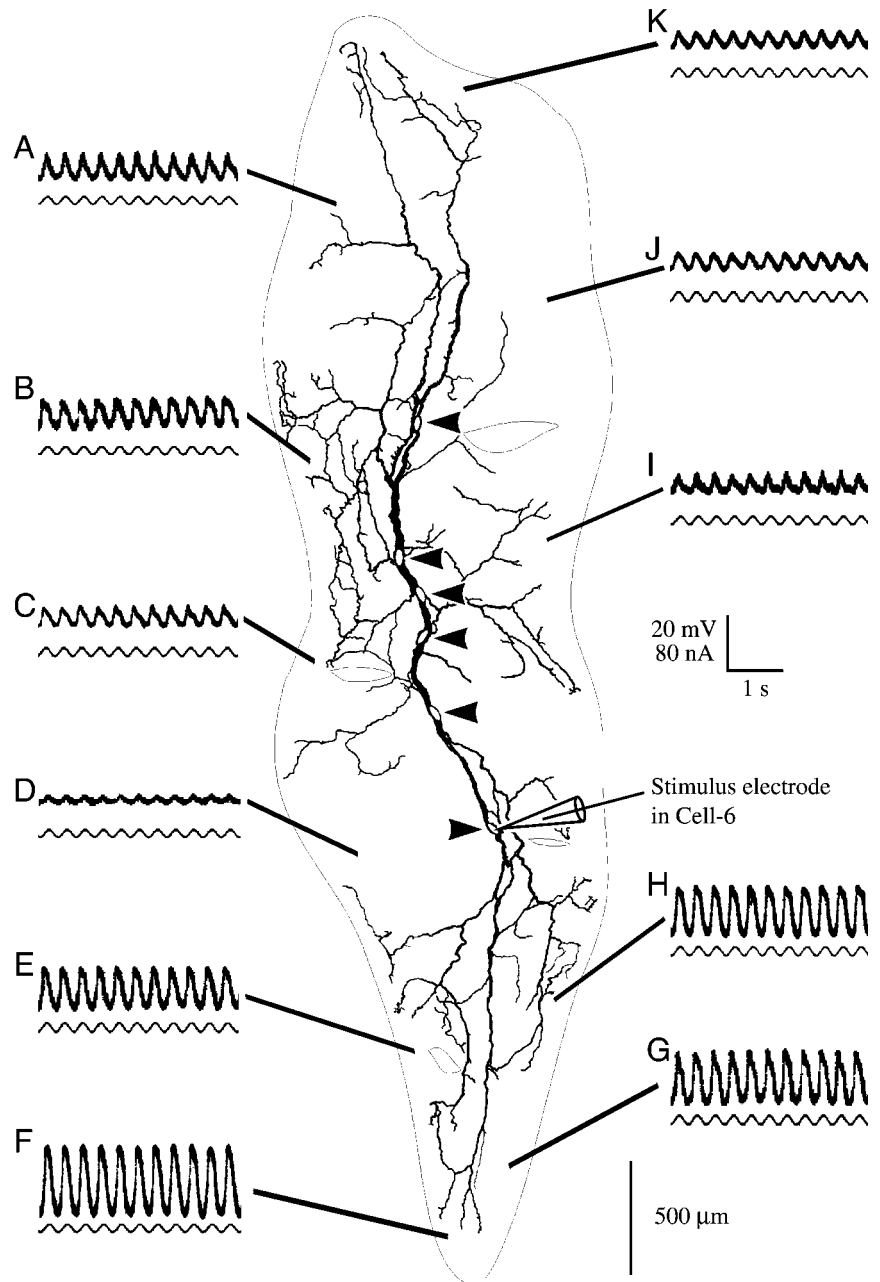


Fig. 3. Effects of sinusoidal current injection into the soma of a CG neurone on myocardial membrane potential. The membrane potentials of myocardial fibres were recorded successively from various points (A–K) on the heart wall in saline containing  $1.0 \mu\text{mol l}^{-1}$  tetrodotoxin (TTX), while a sinusoidal current stimulus was applied continuously to Cell-6 through a microelectrode. The stimulus electrode was filled with 4% neurobiotin in  $1 \text{ mol l}^{-1}$  KCl to stain the CG neurones. The inset drawing shows the stained processes of the CG neurones (anterior is towards the top of the figure). Arrowheads indicate the location of the six somata. In each recording, membrane potential activity of the myocardial fibre (upper trace) and the stimulus current (lower trace) are shown. The resting potentials of the recorded myocardial fibres were in the range  $-38$  to  $-35 \text{ mV}$ . In the absence of stimuli, no spontaneous myogenicity was observed in this preparation.

recorded at various membrane potential levels using a two-electrode voltage-clamp in high-TTX saline. Fig. 5A shows a representative series of membrane current responses of the myocardium to sinusoidal current injected into the soma of a CG neurone in high-TTX saline. When the sinusoidal stimulus was applied to the neurone, periodic bursts of inward currents were evoked overlying the rising phase of the sinusoidal artefact caused by the stimuli. Changing the holding potential in the depolarizing direction decreased the amplitude of the current responses. The relationship between the amplitude of the membrane current responses and the holding potential was linear (Fig. 5B). In the example shown in Fig. 5, the reversal potential for the current responses estimated by extrapolation of the linear relationship was  $+14.1 \text{ mV}$ . The mean value of the

reversal potentials in three preparations estimated similarly by the regression lines was  $+7.1 \pm 2.3 \text{ mV}$  (mean  $\pm$  S.E.M.).

#### *Characterization of graded membrane potential responses of the myocardium*

The relationship between the graded responses of the myocardium and the intensity of the stimuli to a CG neurone was determined. Brief current pulses (0.2 s in duration) of various amplitudes were applied to a CG neurone while the membrane potential responses of the myocardium in high-TTX saline were monitored (Fig. 6). A brief depolarizing or hyperpolarizing pulse to the CG neurone induced an initial depolarizing or hyperpolarizing response and subsequent oscillation in the myocardial membrane potential (Fig. 6Ai,ii).

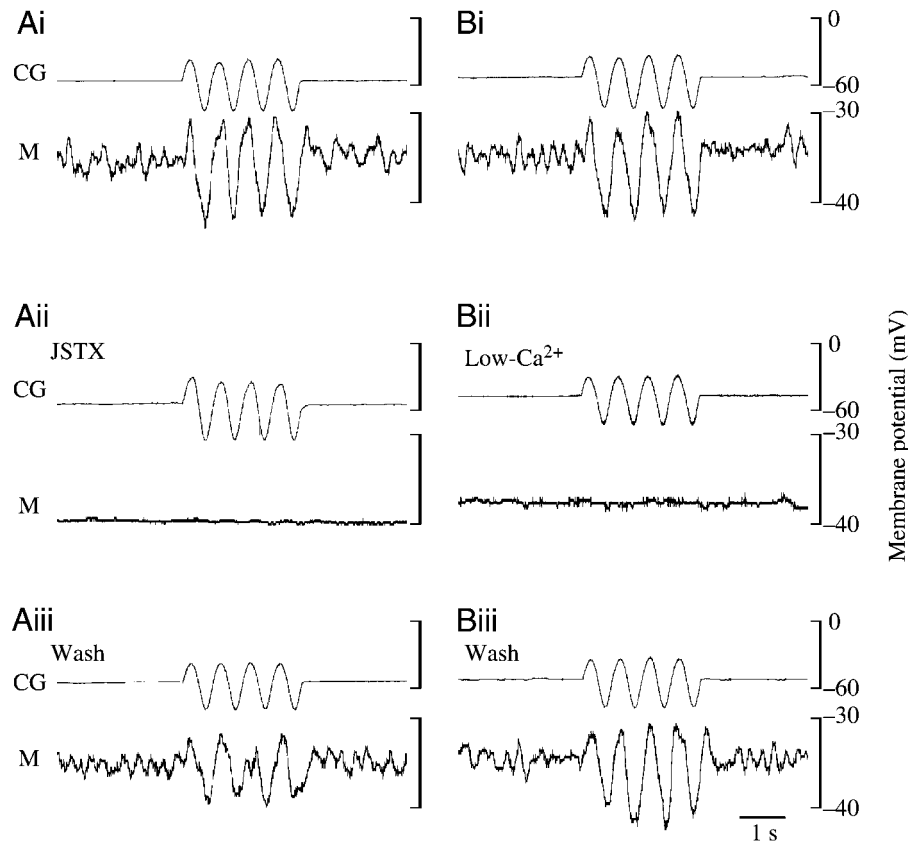


Fig. 4. Effects of Joro spider toxin (JSTX) and low- $\text{Ca}^{2+}$  saline on membrane potential responses of the myocardium to sinusoidal current stimuli applied to a CG neurone. In each recording, the membrane potential of a CG neurone (CG, upper trace) and the myocardial fibre (M, lower trace) were recorded simultaneously in saline containing  $1.0 \mu\text{mol l}^{-1}$  tetrodotoxin (TTX). Sinusoidal current stimuli ( $\pm 15 \text{ nA}$  in amplitude) were applied to the soma of the CG neurone through the recording electrode. Recordings were obtained before (Ai, Bi), 10 min after the addition of JSTX (Aii) or substitution of low- $\text{Ca}^{2+}$  saline (Bii), and after washout of JSTX or low- $\text{Ca}^{2+}$  saline (Aiii, Biii). The sinusoidal stimuli to the CG neurone evoked similar sinusoidal membrane potential responses in the myocardium. The responses were blocked by both JSTX and low- $\text{Ca}^{2+}$  saline. Recordings in A and B were obtained from different preparations.

The latency of the muscle response to the stimulus was in the range 50–70 ms ( $N=52$ ), but could not be determined accurately because the resting potential of the myocardium was unstable in high-TTX saline.

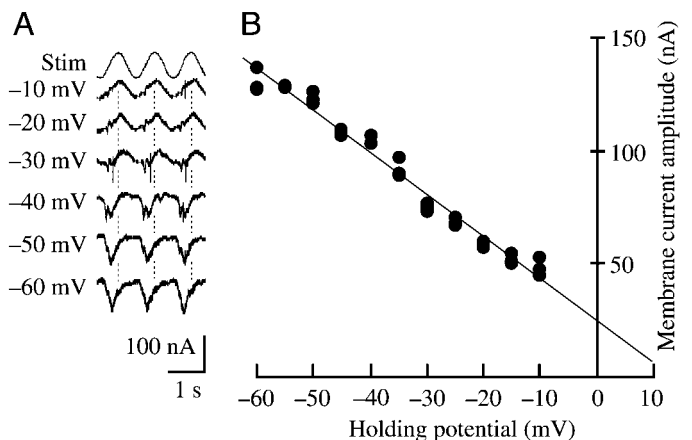
In Fig. 6B, the amplitudes of initial membrane potential responses (mV) of the myocardium are plotted against the amplitudes of the stimulus pulses (nA) applied to the soma of the CG neurone. The membrane potential of the myocardium at the peak of the initial response ranged from  $-57$  to  $-22 \text{ mV}$ , and the relationship was sigmoidal. The resting potential of the myocardium in the absence of CG stimulation was

approximately  $-36 \text{ mV}$ , which is on the steep portion of the sigmoidal curve.

#### Recording of excitatory junctional currents at neuromuscular junctions

Excitatory junctional currents (EJCs) were recorded from a neuromuscular junctional site on the myocardium (Figs 7, 8). An active spot where negative-going junctional currents occur spontaneously was located by careful probing with the macropatch electrode. Fig. 7A shows a representative set of simultaneous recordings of membrane potentials in a CG neurone and a myocardial fibre (top and middle traces) and the extracellularly recorded EJCs (bottom trace) in normal saline.

Fig. 5. Reversal potential of the junctional current response of the myocardium. (A) The junctional currents recorded at various holding potentials using a two-electrode voltage-clamp in saline containing  $1.0 \mu\text{mol l}^{-1}$  tetrodotoxin (TTX). Sinusoidal current ( $\pm 36 \text{ nA}$ , top trace corresponding to the recording at  $-10 \text{ mV}$ ; Stim) was injected into the soma of a CG neurone. The dotted lines indicate peaks of stimulus current. Note that inward (downward) currents occurred on the rising phase of the sinusoidal artefact. The amplitude of the inward current decreased as the holding potential was changed from  $-60$  to  $-10 \text{ mV}$ . The resting potential of the myocardial fibre was  $-36 \text{ mV}$ . (B) The relationship between the holding potential and the junctional current amplitude obtained from a representative preparation. The amplitude of the membrane current responses was obtained by subtracting the instantaneous amplitude of the stimulus from the recorded responses. Three values are plotted against each holding potential. The reversal potential estimated from the linear regression line ( $r^2=0.975$ ,  $P<0.0001$ ) is  $+14.1 \text{ mV}$ .



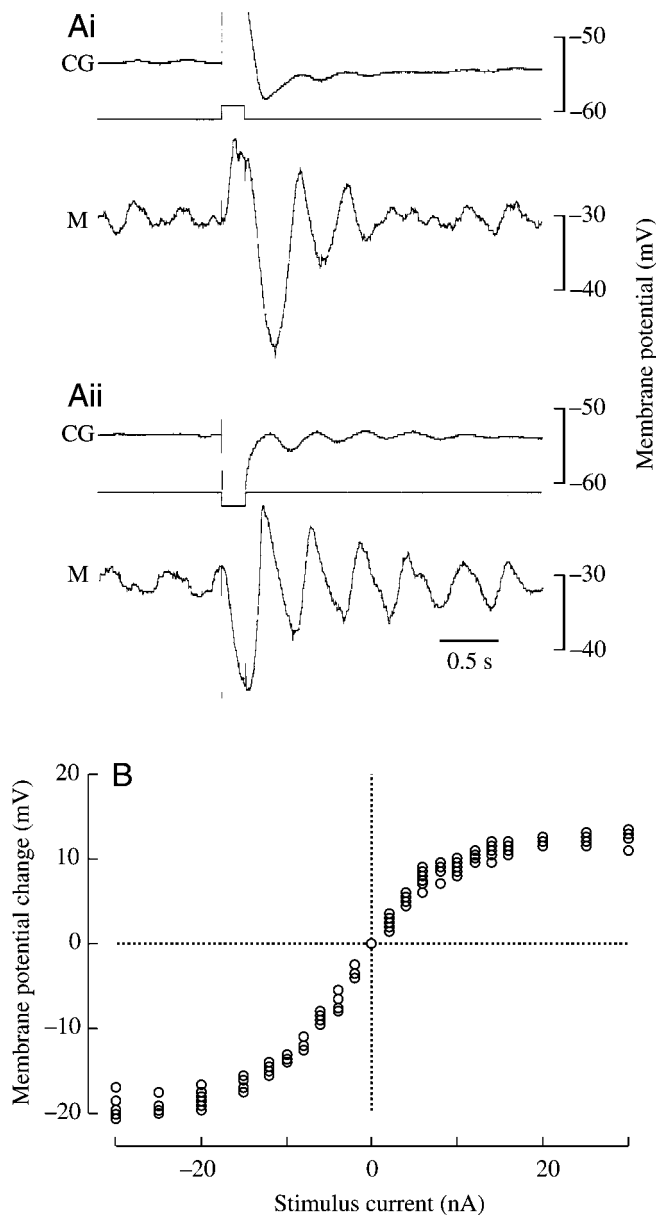


Fig. 6. Relationship between the current stimuli to a CG neurone and the membrane potential responses of the myocardium. (A) Membrane potential responses of a CG neurone (CG) and a myocardial fibre (M) to a square current stimulus applied to the soma of the CG neurone in the presence of  $1.0 \mu\text{mol l}^{-1}$  tetrodotoxin (TTX). A depolarizing (Ai) or hyperpolarizing (Aii) current pulse ( $\pm 15 \text{ nA}$ ,  $0.2 \text{ s}$  in duration) was injected into the soma through the recording electrode. In each recording, the right-hand voltage scale indicates the absolute value of the membrane potential. Note that the myocardium responded to both depolarizing and hyperpolarizing stimuli to the CG neurone. (B) The relationship between the stimulus intensity and the membrane potential response of the myocardium. The amplitudes of the depolarizing or hyperpolarizing responses are plotted against the intensity of the stimulus current. Five responses recorded from one preparation are plotted at each value of the stimulus current.

EJPs and large EJC ( $0.6\text{--}1.1 \text{ nA}$  in amplitude) were evoked in the myocardial fibre when the CG neurone generated spikes

(Fig. 7A). In addition to these spike-evoked EJCs, miniature EJCs asynchronous with the CG spikes of various amplitudes ( $0.04\text{--}0.8 \text{ nA}$ ) were also recorded. The occurrence of these asynchronous EJCs was observed at most of the junctional sites examined (27 out of 31 recording sites in different preparations,  $N=29$ ), but their frequency varied from site to site. Rapid potential changes corresponding to the asynchronous EJCs were not observed in the myocardium even when recorded at higher gain (not shown).

Fig. 7B shows a typical example of EJCs recorded on slower time base. The frequency of the asynchronous EJCs increased gradually during the inter-burst period and decreased during the after-hyperpolarization in the CG neurone (Fig. 7Bi). Although we do not provide enough data for statistical analysis, the frequency of the asynchronous EJCs tended to be higher when the burst frequency of the CG neurones was relatively low. Following application of JSTX ( $10 \mu\text{mol l}^{-1}$ ) for 10 min, the amplitude of the large EJCs decreased to less than  $0.1 \text{ nA}$  and the miniature EJCs became undetectable (Fig. 7Bii). However, JSTX had little effect on the presynaptic nerve impulses recorded by the same macropatch electrode (Sakurai et al., 1998). Both types of EJC recovered partially 30 min after washout of JSTX (Fig. 7Biii).

When spiking activity in the CG neurones was blocked by application of low-TTX saline, the CG neurone and the myocardium exhibited associated slow depolarizing potentials (Fig. 8A, also see Figs 1B, 2B). Under these conditions, miniature EJCs were still observed, increasing or decreasing in frequency in parallel with depolarizing or hyperpolarizing membrane potential changes in the CG neurone (Fig. 8A,Bi). When the CG neurone was made completely quiescent by application of high-TTX saline, the continuous appearance of miniature EJCs was observed (Fig. 8Bii). After washout of TTX, the spike-evoked EJCs and EJPs in the myocardium recovered as the bursting activity of the CG neurones was restored (not shown).

## Discussion

In the present study, we first demonstrated in the opened heart preparation that the periodic bursts of impulses in the CG neurones evoke impulse-mediated EJPs in the myocardium (Figs 1A, 2A) (Yamagishi and Hirose, 1997; Sakurai et al., 1998). In the hearts of many crustaceans, it has long been thought that the motoneurons in the CG generally produce all-or-nothing impulses that propagate along their axons and evoke compound EJPs in the myocardium (for reviews, see Maynard, 1960; Cooke, 1988; Kuramoto and Kuwasawa, 1980). The observations presented here provide several lines of evidence to suggest that the neuromuscular junction in the isopod heart employs a graded mode of synaptic transmission in addition to the impulse-mediated mode.

### Evidence for graded neuromuscular transmission

When impulse generation in the CG was blocked by TTX, the CG neurones and the myocardium often exhibited

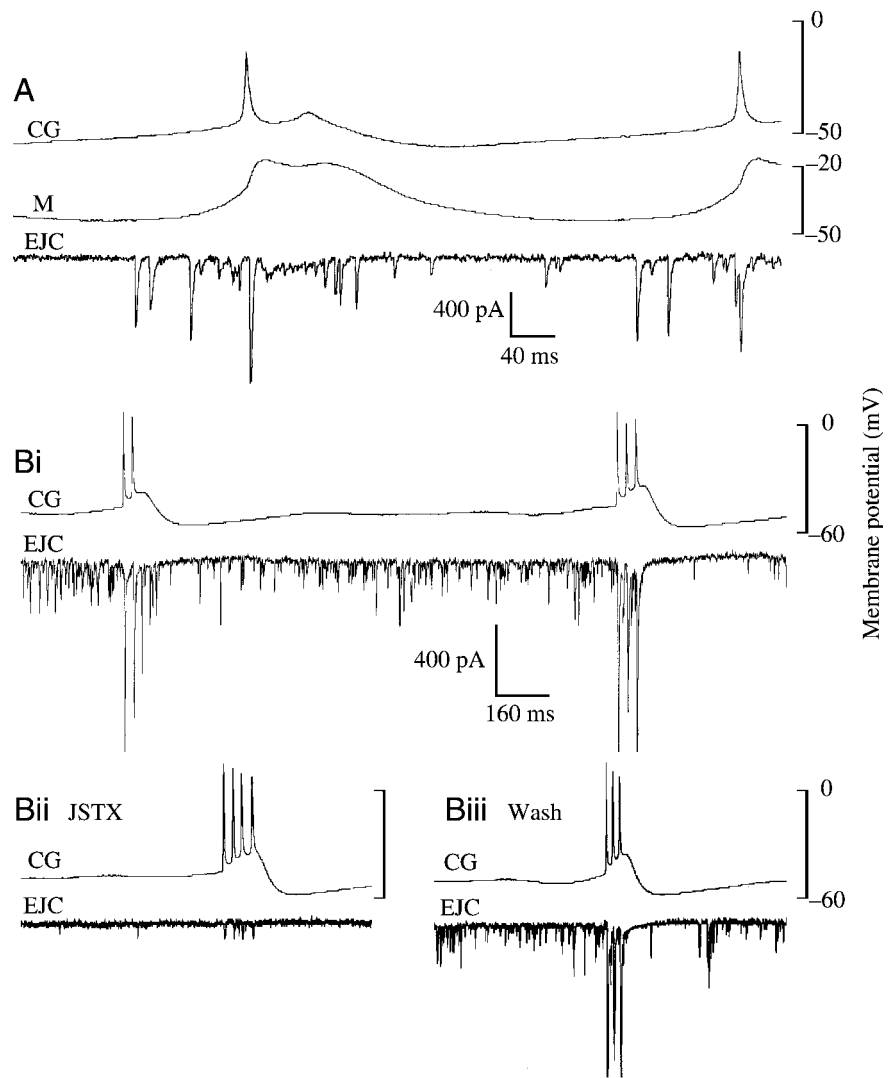


Fig. 7. Extracellular macropatch recordings of junctional currents at a neuromuscular junctional site on the myocardium. (A) Simultaneous recordings of the intracellular activities of a CG neurone (CG, top trace) and the myocardial fibre (M, middle trace) and junctional currents (EJC, bottom trace) recorded extracellularly with a macropatch electrode. In addition to EJCs synchronous with CG spikes, miniature EJCs asynchronous with the CG spikes also occurred. (B) Effects of Joro spider toxin (JSTX) on junctional currents. Simultaneous recordings of the intracellular activity of a CG neurone (CG; upper traces) and the junctional currents (EJC; lower traces) are shown. The recordings were obtained in normal saline (Bi), 10 min after the application of  $10 \mu\text{mol l}^{-1}$  JSTX (Bii) and 30 min after the washout of JSTX (Biii). The right-hand voltage scales indicate the absolute value of the membrane potential. Recordings in A and B were obtained from different preparations.

synchronized membrane activities (Fig. 1B). This non-impulse-mediated coupling of the membrane activities between the CG neurone and myocardium was maintained even when the frequency of the neural activity was experimentally changed by current injection (Fig. 2B). However, it was eliminated by JSTX (Figs 1D, 2C), a specific blocker of glutamatergic neuromuscular transmission (Sakurai et al., 1998). When the CG neurones were made quiescent by application of saline containing a higher concentration of TTX ( $1.0 \mu\text{mol l}^{-1}$ ), a sinusoidal current stimulus applied to the soma of a CG neurone evoked a similar sinusoidal membrane potential change in the myocardial fibres of almost the entire heart wall (Figs 3, 4). The sinusoidal muscle responses were blocked by JSTX (Fig. 4Aii) or low- $\text{Ca}^{2+}$  saline (Fig. 4Bii) and lagged behind the stimulus to the neurone (Fig. 6A). The relationship between the stimulus intensity applied to a CG neurone and the amplitude of the myocardial membrane potential responses was sigmoidal (Fig. 6B). These results suggest that the slow membrane potential activity of the CG neurones was transmitted to the myocardium in the absence of nerve impulses, and that this non-impulse-mediated

transmission was mediated by graded release of a chemical transmitter, probably glutamate, from the CG neurones.

The voltage-clamp experiments revealed that the membrane current responses of the myocardium to stimuli applied to the CG neurone have a reversal potential above  $0 \text{ mV}$  ( $+7.1 \pm 2.3 \text{ mV}$ ; mean  $\pm$  S.E.M.,  $N=3$ ) (Fig. 5). This value is similar to that of the impulse-mediated EJCs ( $+8.3 \pm 2.3 \text{ mV}$ ,  $N=4$ ) obtained in a previous study (Sakurai et al., 1998), supporting the idea that non-impulse-mediated neuromuscular transmission is mediated by the same transmitter, glutamate, as impulse-mediated transmission.

We also recorded the spontaneous appearance of miniature EJCs that were asynchronous with spikes in the recorded CG neurone (Figs 7, 8). It is unlikely that these miniature EJCs were evoked by asynchronous spiking activity in other CG neurones, since it has been shown previously that all six CG neurones exhibit synchronized bursting activities *via* strong electrical connections (Yamagishi and Ebara, 1985). The miniature EJCs were TTX-resistant (Fig. 8), but were blocked by JSTX (Fig. 7Bii), and their frequency increased in parallel with the membrane potential changes in the CG neurone



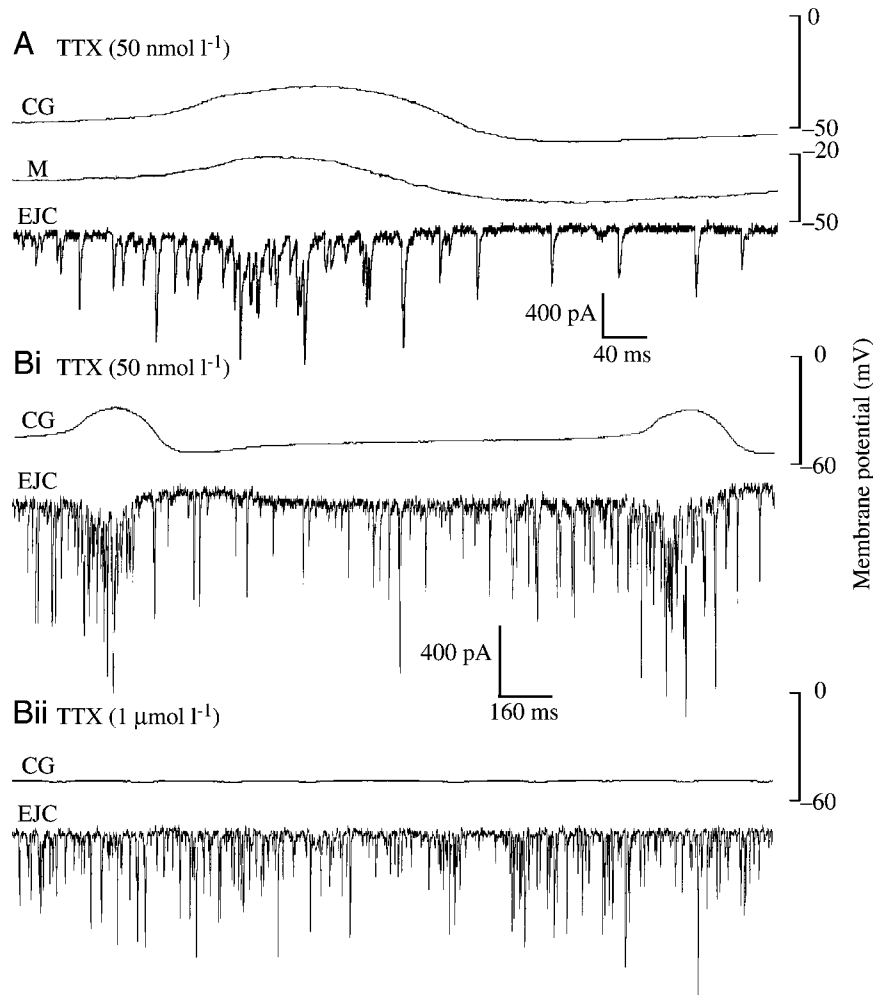


Fig. 8. Effects of tetrodotoxin (TTX) on junctional currents recorded at a neuromuscular junctional site on the myocardium. (A) Intracellular activities of the CG neurone (CG, top trace) and the myocardial fibre (M, middle trace), and the extracellular junctional currents (EJC, bottom trace), were recorded simultaneously in saline containing  $50 \text{ nmol l}^{-1}$  TTX. (B) Effects of TTX at different concentrations on the junctional currents. The intracellular activity of the CG neurone (CG; upper traces) and the junctional currents (EJC; lower traces) were recorded in  $50 \text{ nmol l}^{-1}$  TTX (Bi) and  $1.0 \mu\text{mol l}^{-1}$  TTX (Bii). Note that in  $50 \text{ nmol l}^{-1}$  TTX the CG neurone and the myocardium exhibited coupled membrane potential changes and that the frequency of EJCs changed in parallel with these potential changes. The EJCs appeared continuously even when the CG neurone was quiescent in  $1.0 \mu\text{mol l}^{-1}$  TTX. The right-hand voltage scales indicate the absolute value of the membrane potential. Recordings in A and B were obtained from the same preparation.

(Figs 7Bi, 8). These results strongly support the idea that the CG neurones exhibit non-impulse-mediated release of glutamate, depending on the presynaptic membrane potential changes, in addition to impulse-mediated release.

In this study, we failed to record rapid potential changes in the myocardium corresponding to extracellularly recorded miniature EJCs (Figs 7A, 8A). However, in preparations with less myogenicity, irregular fluctuations of myocardial membrane potential were often observed when ganglionic activity was suppressed by high-TTX saline (Figs 4, 6A). Application of either JSTX or low- $\text{Ca}^{2+}$  saline diminished this membrane potential fluctuation and caused a slight hyperpolarization of muscle resting potential (Fig. 4Aii). Together with the results obtained from macropatch recordings in high-TTX saline (Fig. 8Bii), we assume that these membrane fluctuations in the myocardium were at least in part the miniature junctional potentials caused by random transmitter releases from a large number of CG terminals. It is likely that, in normal and low-TTX saline, spatial and temporal summation of these miniature potentials and overlying myogenicity, rather than rapid discrete potentials, cause the slow overall depolarization in the myocardium.

Since the early proposal of neuronal interaction without

action potentials by Bullock (1959), graded synaptic transmission has been demonstrated in many nonspiking and spiking neurones in central and peripheral ganglia (for a review, see Pearson, 1976). The graded nature of transmitter release depending on presynaptic voltage has also been reported at the excitatory neuromuscular junctions of both vertebrates and invertebrates (rat and frog, Katz, 1962; Katz and Miledi, 1965, 1967a,b; crayfish, Dudel, 1982, 1983; Atwood et al., 1987). However, propagation of axonal impulses is still essential for carrying motor signals from central neural circuits to junctional terminals. Thus, the present results are the first report of neuromuscular transmission that passes both all-or-nothing and graded motor signals from the soma of the motoneurons to the muscles as a part of their normal function.

The results obtained by current injection into a CG neurone in high-TTX saline (Figs 3, 4, 6) suggest that the stimulus-induced membrane potential change in the soma of the neurone spreads to the presynaptic terminals to cause transmitter release. The spread of slow electrical signals in CG axons is likely to be electrotonic since the amplitude of the muscle response became smaller at sites more distant from the stimulated neurone (Fig. 3A–C, I–K). However, we could not

exclude the possibility that voltage-dependent membrane responses in the soma and the myocardium were also involved in these responses. In decapod CG neurones, slow membrane potential changes or so-called 'driver potentials' in the large cells are produced at the proximal regions near the somata and spread electrotonically along the axons (Tazaki and Cooke, 1979b, 1983a,b). In *Ligia exotica*, we assume that the CG neurones have junctional terminals near their somata and the CG trunk region and/or that the length constant of the CG axons is long enough to convey signals to their terminals electrotonically. In this study, we could not determine the length constant of the axon of the CG neurones because of the difficulty of recording intracellularly from their axons. The relationship between the extent of electrotonic spread in the CG neurones and the distribution of the junction sites exhibiting graded transmission should be examined further.

#### *Characteristics and functional role of graded neuromuscular transmission*

The myocardium exhibited not only depolarizing responses, but also hyperpolarizing responses to the depolarizing and hyperpolarizing currents injected into a CG neurone in high-TTX saline (Figs 4, 6). In the graph showing the stimulus-response relationship (Fig. 6B), the resting potential of the myocardium lies on the steep portion of the sigmoidal curve. This result suggests that, when the CG neurones are quiescent in high-TTX saline, the membrane potential of the myocardium is held at a somewhat depolarized level from its resting potential. This idea is further supported by the results of the macropatch recordings. In Figs 7 and 8, we observed that the frequency of miniature EJCs changed with changes in the membrane potential of the CG neurone. When the CG neurones were quiescent in high-TTX saline, miniature EJCs were still observed continuously (Fig. 8Bii). These observations suggest that the CG neurones release transmitter continuously at their resting potential level in high-TTX saline, keeping the myocardium at a slightly depolarized level. Furthermore, the slow membrane potential changes in the CG neurone would cause changes in the amount of transmitter released and consequently induce overall membrane potential changes in the myocardium. Such changes in the myocardium may trigger additional myogenic activity (see oscillatory after-potentials in Fig. 6A) due to the voltage-dependent conductance of the myocardial membrane.

We conclude that slow membrane potential changes in CG neurones can induce membrane potential changes in the myocardium by changing the amount of tonic transmitter release from the CG neurones. Under normal conditions, the CG neurones exhibit periodic slow depolarizing potentials that give rise to bursts of action potentials followed by after-burst hyperpolarization (Fig. 7A) (Yamagishi and Ebara, 1985). Slow depolarizing potentials are commonly seen in many bursting neurones in crustacean cardiac and stomatogastric ganglia (for a review, see Benson and Cooke, 1984), and they are thought to be essential for the formation of periodic bursts of action potentials (Tazaki and Cooke, 1979a,b). From the

results obtained in the present study, we suggest that the slow membrane potential changes such as the plateau potential and the after-burst hyperpolarization in the CG neurones of *Ligia exotica* have two important roles in relation to their pacemaker and motoneuronal functions: (i) to generate periodic bursts of all-or-nothing impulses that consequently evoke bursts of EJPs in the myocardium, and (ii) to produce the overall graded potentials in the myocardium *via* non-impulse-mediated synaptic transmission. Thus, using digital and analogue modes of transmission, the CG neurones firmly entrain the myogenic activity of the myocardium in *Ligia exotica*.

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