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GLUTAMATERGIC NEUROMUSCULAR TRANSMISSION IN THE HEART OF THE ISOPOD CRUSTACEAN *LIGIA EXOTICA*

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Summary

Neuromuscular transmission between the cardiac ganglion (CG) and the myocardium was examined in the adult heart of the isopod crustacean *Ligia exotica*. Intracellular injection of neurobiotin into the CG neurones revealed that all six CG neurones send their axons onto the cardiac muscle, where they form axon terminals bearing varicosities. All the CG neurones and their processes exhibited glutamate-like immunoreactivity. The cardiac muscle showed depolarizing membrane potential responses to glutamate applied focally to sites close to axon terminals bearing varicosities. Both the glutamate-induced response and the excitatory junctional potential (EJP) showed desensitization in response to the repeated application of glutamate. Under voltage-clamp conditions, the cardiac muscle produced inward current responses to focally applied glutamate. The reversal potential for the glutamate-induced current estimated from extrapolation of the linear current/voltage relationship was similar to that of the excitatory junctional current evoked by ganglionic nerve stimulation. Both the glutamate-induced response and the EJP were blocked by a glutamate-specific antagonist, Joro spider toxin. These results led us to conclude that the CG neurones of *Ligia exotica* are glutamatergic motoneurones.

Key words: *Ligia exotica*, Crustacea, heart, glutamate, neuromuscular transmission.

Introduction

The hearts of many crustaceans are known to be neurogenic. The myocardium has no inherent automaticity, and the cardiac ganglion (CG) acts as the dominant pacemaker of the heartbeat. The bursting activity of the CG produces periodic bursts of excitatory junctional potentials (EJPs) in the cardiac muscle and causes its periodic contraction, the neurogenic heartbeats (reviewed by Krijgsman, 1952; Maynard, 1960; Prosser, 1973).

Neuromuscular transmission between the CG and myocardium has been investigated in several crustaceans. Depolarizing membrane potential responses of the cardiac muscle to bath-applied glutamate have been reported in decapods (*Homarus americanus*, Hallet, 1971; *Portunus sanguinolentus*, Benson, 1981) and in isopods (*Porcellio dilatatus*, Holley and Delaleu, 1972; *Bathynomus doederleini*, Yazawa *et al.* 1990). In addition, the cardiac ganglion neurones of the isopod *Bathynomus doederleini* have been confirmed to be glutamatergic by pharmacological experiments and by high-performance liquid chromatography (Yazawa *et al.* 1997, 1998; reviewed by Tanaka *et al.* 1996). However, on the basis of the results of pharmacological experiments, dopamine has been suggested to be the transmitter at the neuromuscular junction between the CG and myocardium in the heart of the hermit crabs *Aniculus aniculus* and *Dardanus classimanus* (Yazawa and Kuwasawa, 1992).

Recently, Yamagishi and Hirose (1997) reported that the heart pacemaker of the isopod *Ligia exotica* is transferred from the myocardium to the CG during juvenile development and that the heartbeat changes from myogenic to neurogenic. The CG of *Ligia exotica* consists of six CG neurones (Suzuki, 1934). Each CG neurone has pacemaker properties and has been suggested to be a motoneurone innervating the myocardium (Yamagishi and Ebara, 1985). The CG begins to generate periodic bursting activity during juvenile development and becomes a primary pacemaker to entrain the myogenic activity *via* EJPs. This type of late-developing neurogenic heartbeat is different from the neurogenic heartbeats known in any other crustacean.

In the present study, we examined the projection of the CG neurones onto the myocardium and neuromuscular transmission between the CG and myocardium in the heart of adult *Ligia exotica* using morphological, immunohistochemical, electrophysiological and pharmacological methods. The results suggest that all the CG neurones are motoneurones innervating the myocardium and are glutamatergic. Some of these results have appeared in

abstract form (Sakurai and Yamagishi, 1996, 1997; Mori *et al.* 1997).

Materials and methods

Adult males and females of the littoral isopod *Ligia exotica* (Roux), 15–30 mm in body length, were used. They were collected at Pacific seashores (at Izu and Boso, Japan) and kept in the laboratory at room temperature (22–26 °C). Over 100 specimens were used for the experiments.

The anatomy of the heart was detailed by Yamagishi and Ebara (1985). The heart tube consists of a single layer of striated muscle fibres, and the CG is located on the inner surface of the dorsal heart wall. The CG consists of six similarly sized neurones (*Ligia exotica*, Suzuki, 1934; *Ligia oceanica*, Alexandrowicz, 1952) whose somata lie longitudinally along the CG trunk (Fig. 1A). All the CG neurones have pacemaker properties and fire synchronously through electrical interactions (Yamagishi and Ebara, 1985). Periodic bursts of impulses generated in the CG are conducted peripherally *via* the ganglionic nerves and produce periodic bursts of EJPs in the cardiac muscle (Yamagishi and Hirose, 1997).

Preparations and physiological salines

Isolated heart preparations were used for the experiments. The heart was opened by longitudinal incision of the ventral heart wall and was pinned in the experimental chamber with its inner surface uppermost. In some cases, the nervous system in the heart was stained by perfusion with saline containing Methylene Blue (0.05 %) for 30–60 min prior to experimentation. During experiments, the preparation was perfused with aerated physiological saline of the following composition (in mmol l^{-1}): NaCl, 577; KCl, 14; $CaCl_2$, 25; $MgCl_2$, 21; Na_2SO_4, 4.5; and Tris, 5 (Yamagishi and Ebara, 1985). The pH was adjusted to 7.4 using HCl. In some experiments, Ca^{2+}-free saline was prepared by replacing $CaCl_2$ with $MgCl_2$ to block chemical synaptic transmission. Experiments were performed at room temperature (22–26 °C).

The drugs employed in this study were as follows: L-glutamate, quisqualic acid, kainic acid, dopamine, acetylcholine and Joro spider toxin (JSTX). Quisqualic acid was obtained from Sigma Chemical Co., and the other chemicals were obtained from Wako Pure Chemicals. Each drug was 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 the intracellular activities of the cardiac muscle and CG neurones. Compound EJPs were evoked in the cardiac muscle by stimulating the distal cut end of the ganglionic nerve with a suction electrode. To obtain EJPs of constant amplitude, unitary EJPs were evoked by adjusting the stimulus strength to the axon with the lowest threshold or by intracellular injection of square current pulses (20–50 ms in duration) into the soma of the CG neurone. Spontaneous excitatory junctional currents (EJCs) were recorded extracellularly using a macropatch electrode (e.g. Dudel and Kuffler, 1961*a*,*b*; Cooper *et al.* 1995*a*,*b*). The tip of a microelectrode was bent and heat-polished (inner diameter, 10–20 μm). This macropatch electrode was filled with physiological saline and connected to the current–voltage converter with a feedback resistance of 200 MΩ. EJCs were recorded by placing the macropatch electrode onto the neuromuscular junctional site on the cardiac muscle. The resistance of the macropatch electrode measured by passing square current pulses was 0.5–1.0 MΩ. The seal resistance between the electrode and the muscle was 0.3–1.0 MΩ.

To determine the reversal potentials for the glutamate-induced current and EJC, the membrane of the cardiac muscle was clamped at various potentials 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 muscle fibre (approximately 30–75 μm wide and 1.5–3.3 mm in length). The length constant along the single muscle fibre was 586.9±26.8 μm (mean ± S.E.M., N=8) and that across the contiguous fibres was 433.2±23.9 μm (mean ± S.E.M., N=6). The distance between the two electrodes

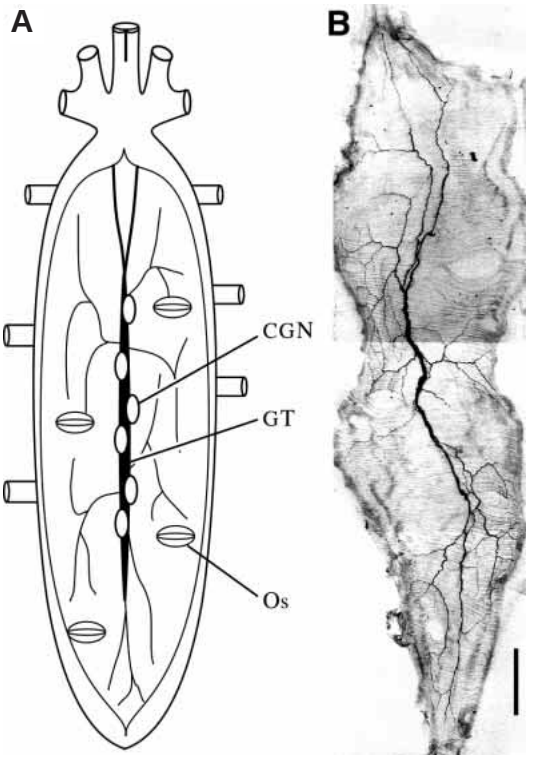

Fig. 1. The cardiac ganglion (CG) of *Ligia exotica*. (A) Schematic view of the inner dorsal wall of the heart. The CG lies along the longitudinal midline of the dorsal heart wall. CGN, cardiac ganglion neurone; GT, ganglionic trunk; Os, ostium. (B) Light micrograph of the CG system. All six CG neurones and their axons have been labelled with neurobiotin injected intracellularly into the soma of one CG neurone. Scale bar, 500 μm.

inserted into the same single muscle fibre was less than 100 μm. Although the membrane potential of the whole single muscle fibre could not be clamped, the membrane potential changes caused by current injection deviated by less than 10 % between the sites impaled by the microelectrodes.

The signals were stored on an FM tape data-recorder, displayed on the chart recorder or on the cathode ray tube and photographed.

Focal application of glutamate

Focal application of glutamate was made *via* a micropipette with a tip diameter of less than 5 μm. The pipette was bent near its tip and filled with 1 mmol l^{-1} glutamate in Ca^{2+}-free saline. In some cases, the pipette was filled with 1 mmol l^{-1} quisqualic acid or kainic acid in Ca^{2+}-free saline. The pipette was connected to Picospritzer (General Valve Co.) by a plastic tube. Glutamate was applied focally to the cardiac muscle for 20–50 ms by pressure ejection. To identify the glutamate-sensitive sites in the cardiac muscle, the tip of the pipette was positioned close to the cardiac muscle and was moved slowly along the muscle fibre while applying glutamate repeatedly at 0.1 Hz. When the cardiac muscle showed a depolarizing response to applied glutamate, the pipette was lifted slightly to prevent desensitization of the receptors. In the experiments under voltage-clamp conditions, glutamate was applied focally to the region between the electrodes, so that the glutamate-induced current would flow through the clamped area of the muscle membrane.

Intracellular labelling with neurobiotin

The somata and axons of the CG neurones were visualized by intracellular injection of neurobiotin [*N*-(2-aminoethyl) biotinamide hydrochloride; Vector Laboratories]. A microelectrode (resistance, 20–40 MΩ) filled with neurobiotin solution (4 % in 1 mol l^{-1} KCl) was inserted into the soma of a CG neurone and current pulses (positive square pulses of 5–10 nA at 50 % duty cycle) were applied at a frequency of 1–2 Hz for 30–60 min. The heart was then incubated in aerated physiological saline for 10 h at room temperature. After the incubation period, the heart was fixed in 4 % paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS, pH 7.4) for 5 h at 4 °C. After several rinses with PBS, the heart was treated with Triton X-100 (2.0 % in PBS) overnight, with H_2O_2 (0.3 % in distilled water) for 1 h, and then incubated in avidin–biotin–peroxidase complex (Vectastain ABC-elite kit, Vector Laboratories) with 0.3 % Triton X-100 in PBS (PBT) for 3 days at 4 °C. The heart was rinsed several times with PBS and then reacted with 0.02 % diaminobenzidine (Sigma) and 0.006 % H_2O_2 in 0.05 mol l^{-1} Tris buffer (pH 7.4). After rinsing with distilled water, the heart was dehydrated in a graded ethanol series, cleared in methyl salicylate and mounted on a glass slide for light microscopy.

Immunohistochemistry

To examine glutamate-like immunoreactivity in the adult heart of *Ligia exotica*, the heart was opened, keeping it attached to the dorsal carapace, and fixed for 1 h with 4 % paraformaldehyde in sodium carbonate buffer (pH 10–11). The heart was then isolated from the carapace and washed with PBS. The heart was exposed to 5 % normal goat serum and 1 % Triton X-100 in PBS to reduce background staining. Subsequently, the heart was incubated for 1 h at room temperature with a 1:1000 dilution of rabbit anti-glutamate polyclonal antibody (Chemicon) in PBT. After washing with

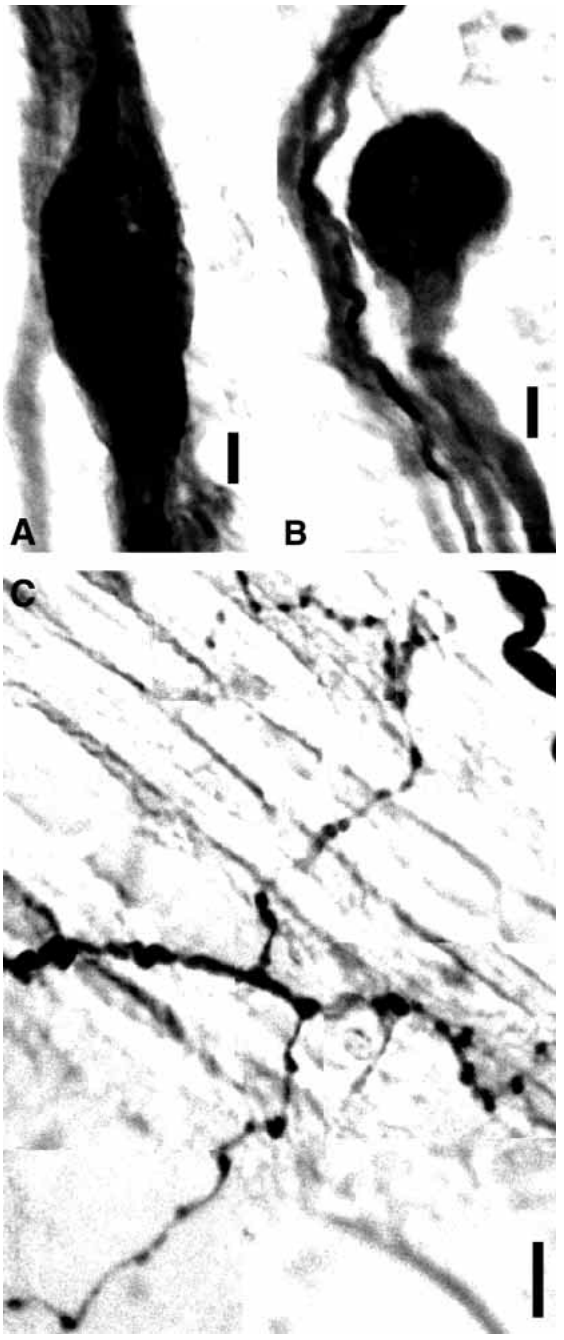

Fig. 2. Light micrographs of the cardiac ganglion (CG) system of *Ligia exotica* labelled with neurobiotin. (A,B) Somata and axons of the CG neurones. Two types of CG neurone, bipolar (A) and monopolar (B), are shown. (C) Varicosities in the axon terminals of the CG neurones. Scale bars, 20 μm.

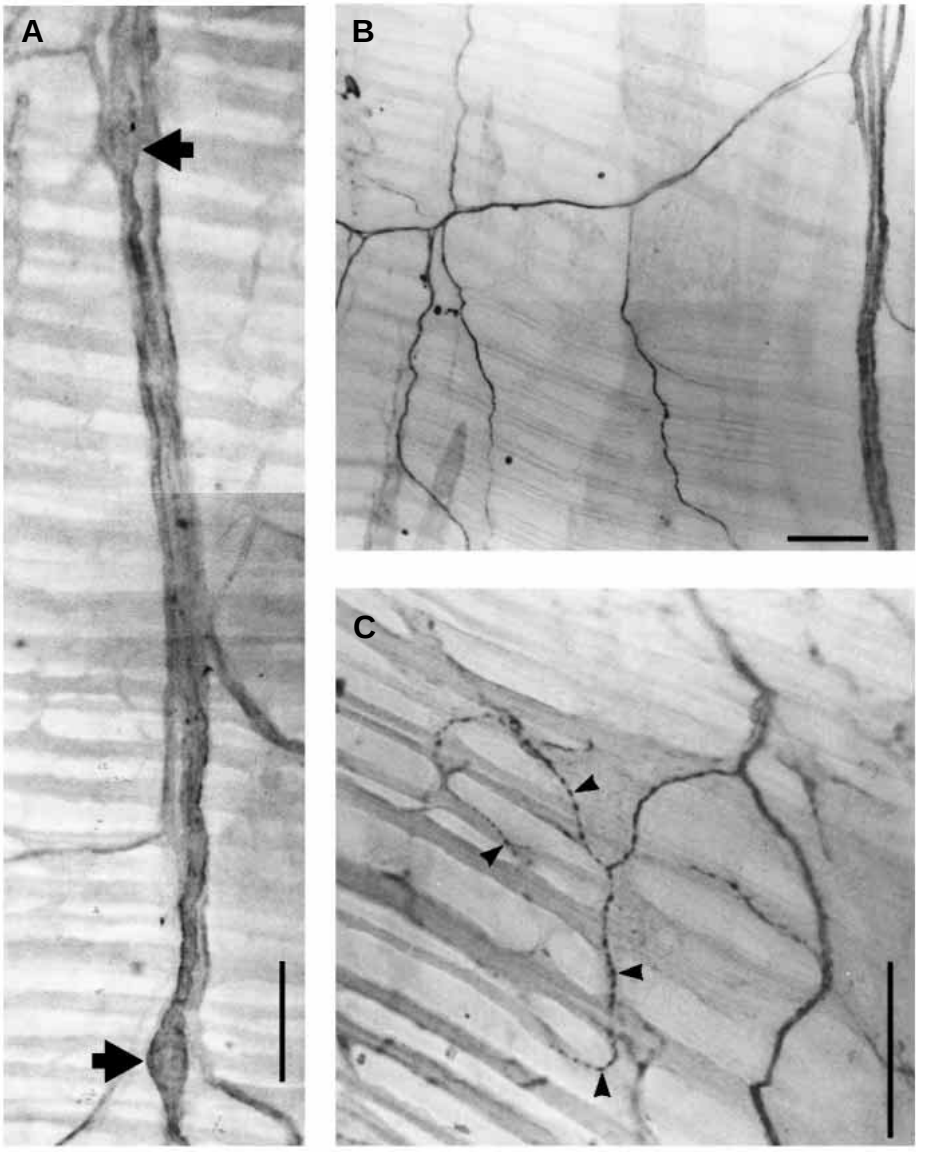

Fig. 3. Glutamate-like immunoreactivity in the heart of *Ligia exotica*. (A) Immunoreactivity in the cardiac ganglionic trunk. Somata (arrows) and axons of the cardiac ganglion neurones are stained. (B) Immunoreactivity in the ganglionic nerves. (C) Immunoreactivity in varicosities (arrowheads) in the axon terminals. Scale bars, 100 μm.

PBT, the heart was incubated with a 1:1200 dilution of biotinylated anti-rabbit IgG (H+L) secondary antibody (Vector Laboratories) in PBT for 1 h and then with avidin–biotin–peroxidase complex (Vectastain ABC-elite kit, Vector Laboratories) for 1 h. The subsequent treatment of the heart was as described above. No immunoreactivity was detected in a negative control preparation incubated without the primary antibody.

Results

Projections of CG neurones onto the myocardium

We examined the morphology of the CG neurones using intracellular injections of neurobiotin. Tracer molecules injected into the soma of any CG neurone spread among all the CG neurones, with the degree of spread depending on the period of injection. The soma and axons of all six CG neurones were stained in 12 preparations (Fig. 1B). In these preparations, the axons of each of the CG neurones could be identified and traced to the peripheral region. The CG neurones were mostly of the bipolar type (Fig. 2A), although monopolar cells were found occasionally (Fig. 2B). The axons (7–18 μm in diameter) of the CG neurones run along the ganglionic trunk and spread diffusely over the inner surface of the heart wall, forming axon terminals bearing many varicosities (Fig. 2C).

Glutamate-like immunoreactivity in the heart was examined in 22 preparations. Fig. 3 shows representative results. Immunoreactivity was found in the somata of the CG neurones, in their axons in the ganglionic trunk (Fig. 3A) and in the nerves deriving from the ganglionic trunk (Fig. 3B). Fine nerve branches and axon terminals bearing varicosities also exhibited glutamate-like immunoreactivity (Fig. 3C).

Effects of glutamate on the myocardium

We examined the effects of several potential transmitters on the cardiac muscle using a bath-application method. To

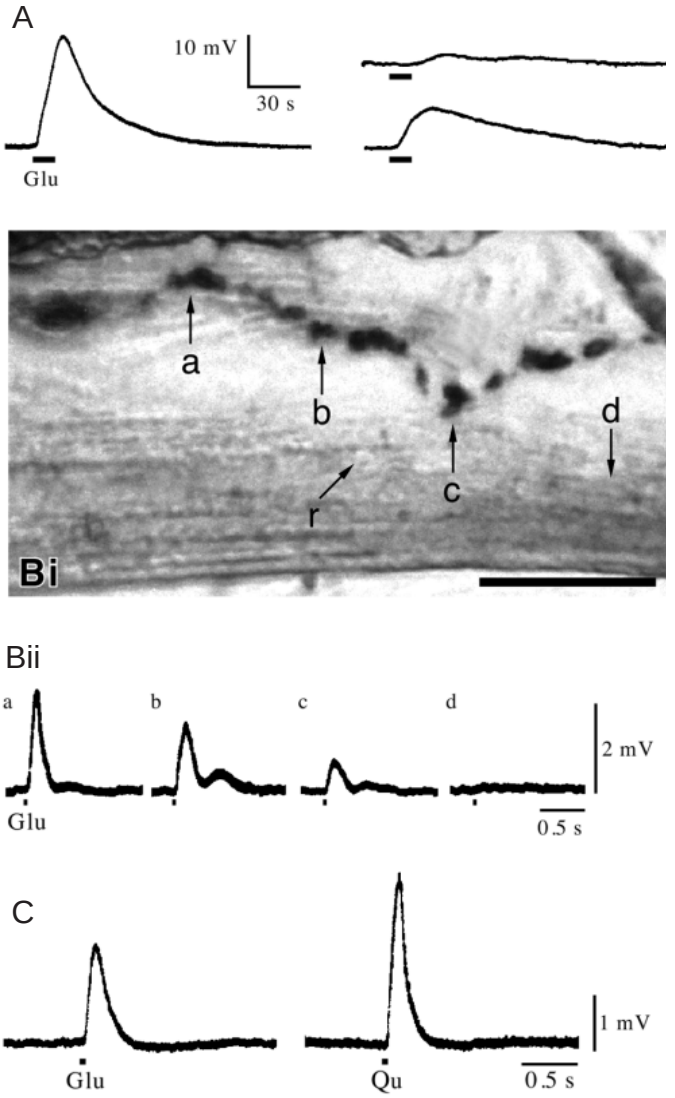

Fig. 4. Effects of glutamate on the myocardium of *Ligia exotica*. (A) Membrane potential responses of the cardiac muscle to glutamate (Glu, 1 mmol l^{-1}) bath-applied during the period indicated by the bar under each recording. The responses were obtained from three different sites of a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −36 mV. (Bi) Light micrograph of the nerve terminal stained with Methylene Blue. The recording site (arrow, r) of muscle responses and the sites (arrows, a–d) of focal application of glutamate (1 mmol l^{-1}) are shown. Scale bar, 40 μm. (Bii) Membrane potential responses of the cardiac muscle to glutamate (Glu, 1 mmol l^{-1}) applied focally at the sites (a–d) shown in Bi during the period indicated by the bar under each recording. From a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −30 mV. (C) Membrane potential responses of the cardiac muscle to glutamate (Glu, 1 mmol l^{-1}) and quisqualic acid (Qu, 1 mmol l^{-1}) applied at the same site during the period indicated by the bar under each recording. From a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −30 mV.

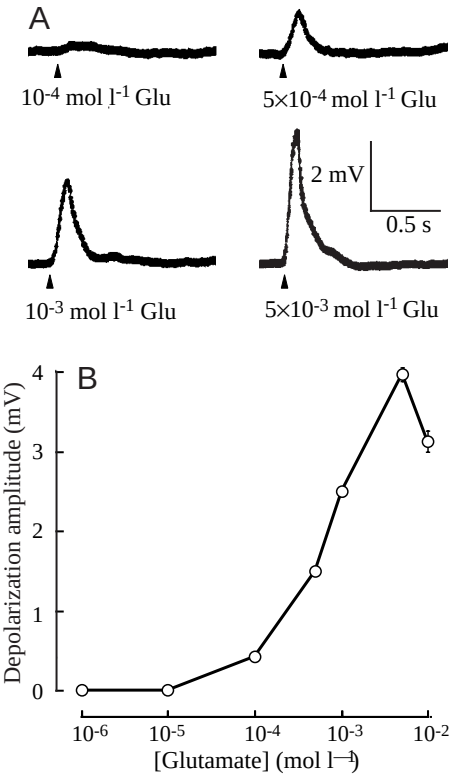

Fig. 5. Dose–response relationship between glutamate and the muscle response. (A) Membrane potential responses of cardiac muscle to glutamate (Glu) applied focally to the same site at various concentrations. From a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −34 mV. (B) Relationship between the concentration of focally applied glutamate and the amplitude of the depolarizing muscle response. Values are means ± S.E.M., *N*=3. For most points, the error bars are smaller than the symbols.

measure the membrane potential responses of the cardiac muscle to these drugs, the heart was perfused with Ca^{2+}-free saline to suppress chemical synaptic transmission. Fig. 4A shows representative results (*N*=13 hearts). Glutamate (1 mmol l^{-1}) induced a depolarizing membrane potential response in the cardiac muscle (Fig. 4A, left). The response declined slowly after washout of glutamate. The amplitude of the response varied from site to site in the same heart (Fig. 4A, right), suggesting localization of the glutamate-sensitive sites in the heart. The other transmitter candidates examined (1.0 mmol l^{-1} dopamine and 1.0 mmol l^{-1} acetylcholine) induced no detectable membrane potential responses in the cardiac muscle (results not shown).

To identify the glutamate-sensitive sites in the heart, focal application of glutamate (1 mmol l^{-1}) was performed in Ca^{2+}-free saline. Prior to experiments, the nerve terminals on the cardiac muscle were stained with Methylene Blue (*N*=3) (Fig. 4Bi). Glutamate applied focally onto sites along the nerve terminal induced depolarizing membrane potential responses in the cardiac muscle (*N*=18) (Fig. 4Bii, site a). The amplitude and the time course of the response changed as the site of glutamate application was moved along the nerve terminal (Fig. 4Bii, sites b,c). When glutamate was applied focally onto a site distant from a nerve terminal, no membrane potential responses were induced in the cardiac muscle (Fig. 4Bii, site

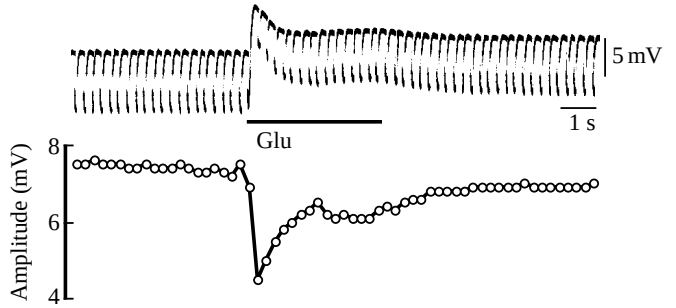

Fig. 6. Membrane resistance changes in the myocardium of *Ligia exotica* induced by glutamate. The upper trace shows the membrane potential recorded intracellularly from the cardiac muscle. Hyperpolarizing current pulses (50 nA, 50 ms) were injected at 4.5 Hz into the muscle *via* a current electrode. Glutamate (Glu, 1 mmol l^{-1}) was applied focally during the period indicated by the bar under the recording. From a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −32 mV. The lower trace shows changes in the amplitude of the hyperpolarizing potential caused by an injected current pulse.

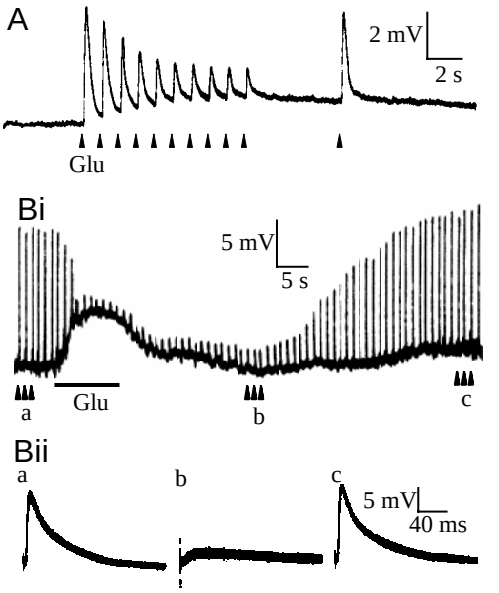

Fig. 7. Desensitization of glutamate and junctional receptors. (A) Membrane potential responses of cardiac muscle of *Ligia exotica* to repeatedly applied glutamate. Glutamate (Glu, 1 mmol l^{-1}) was applied focally nine times at 1 Hz and then (after 5 s) once more to the same site. Arrowheads indicate the times of glutamate application. From a quiescent heart in Ca^{2+}-free saline. Muscle resting potential was −32 mV. (Bi) Effects of bath-applied glutamate on excitatory junctional potentials (EJPs). Unitary EJPs were evoked in normal saline by ganglionic nerve stimulation at 1 Hz. Glutamate (Glu, 1 mmol l^{-1}) was bath-applied during the period indicated by the bar under the recording. Muscle resting potential was −49 mV. (Bii) EJPs recorded at a faster sweep at the times indicated in Bi (arrowheads, a–c). Three sweeps are superimposed in each recording.

d). Thus, the glutamate-sensitivity of the cardiac muscle appeared to be localized to sites close to nerve terminals bearing varicosities.

The effects of glutamate agonists (quisqualic acid and kainic acid) on the glutamate-sensitive sites on the cardiac muscle were also examined using the focal application method (Fig. 4C). Quisqualic acid (1 mmol l^{-1}) induced a depolarizing membrane potential response in the cardiac muscle (Fig. 4D, right) that was larger in amplitude than that in response to glutamate (Fig. 4D, left) (N=5). Kainic acid (1 mmol l^{-1}) induced no detectable membrane potential responses in the cardiac muscle (N=3) (results not shown).

To obtain a dose–response relationship between glutamate and the depolarizing membrane potential responses in the cardiac muscle, glutamate was applied focally to the same spot at various concentrations. The cardiac muscle depolarized in response to the application of 100 μmol l^{-1} to 5 mmol l^{-1} glutamate (Fig. 5A). The amplitude of the responses increased with increasing glutamate concentration. Fig. 5B shows the changes in amplitude of the glutamate-induced response (mean ± S.E.M., N=3) plotted as a function of glutamate concentration. The threshold concentration of glutamate was in the range 10–100 μmol l^{-1}, and the amplitude of the response reach a maximum at 5 mmol l^{-1} glutamate.

Changes in the membrane resistance of the cardiac muscle associated with the glutamate-induced response were examined by injecting brief hyperpolarizing current pulses (50 nA, 50 ms, 4.5 Hz) into the cardiac muscle *via* a second electrode (N=6) (Fig. 6). Focal application of glutamate (1 mmol l^{-1}) for 3.4 s induced a depolarizing membrane potential response in the cardiac muscle, which declined in amplitude during the application period (Fig. 6, upper trace). During the initial phase of the depolarizing response, the muscle membrane resistance decreased to approximately 60 % of the control value and then recovered gradually. The recovery was associated with a decrease in the amplitude of the depolarizing response (Fig. 6, lower trace).

Desensitization of glutamate-induced and junctional responses by glutamate

To determine the desensitization properties of the glutamate-induced response of the cardiac muscle, we examined the effects of repeated focal application of glutamate to the same glutamate-sensitive site (N=6). Focal application of glutamate at 1 Hz caused the amplitude of the depolarizing response of the cardiac muscle to decrease progressively (Fig. 7A). When a glutamate pulse was applied 5 s after the termination of the repetitive application, the amplitude of the response recovered to approximately 75 % of the control value (Fig. 7A). These results suggest that the glutamate receptors on the cardiac muscle exhibit desensitization to glutamate.

We next examined the effects of bath-applied glutamate on EJPs in the cardiac muscle (N=4). Unitary EJPs were evoked repeatedly by stimulation of a ganglionic nerve branch at 1 Hz in normal saline (Fig. 7Bi,ii, trace a). Bath-application of glutamate (1 mmol l^{-1}) caused sustained depolarization in the cardiac muscle and a rapid decrease in the amplitude of the evoked EJPs (Fig. 7Bi). Upon washout of glutamate, the membrane potential

of the cardiac muscle recovered to the control value, but the amplitude of the evoked EJPs at first remained low (Fig. 7Bi,ii, trace b) and then recovered gradually to the control amplitude (Fig. 7Bi,ii, trace c). These results suggest that the junctional receptors at the neuromuscular junctions between the CG and the cardiac muscle were desensitized by bath-applied glutamate.

Reversal potentials for glutamate-induced and junctional currents

To compare the reversal potential for the glutamate-induced responses of the cardiac muscle with that for the junctional responses evoked by ganglionic nerve stimulation, a two-electrode voltage-clamp technique was employed. Fig. 8 shows a representative series of glutamate-induced currents

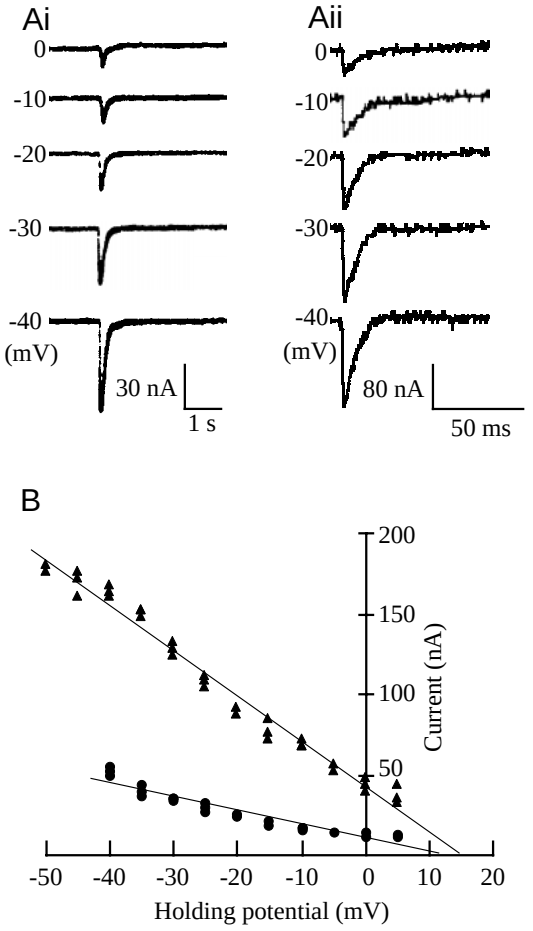

Fig. 8. Reversal potentials of glutamate-induced current and excitatory junctional current (EJC) of *Ligia exotica*. (Ai) Glutamate-induced currents recorded at various holding potentials using the two-electrode voltage-clamp technique. Glutamate (1 mmol l^{-1}) was repeatedly applied focally at the same site. From a quiescent heart in Ca^{2+}-free saline. Resting potential was −32 mV. (Aii) EJCs recorded at various holding potentials. Unitary EJCs were evoked by stimulation of a ganglionic nerve branch in normal saline. Resting potential was −27 mV. (B) Relationship between the holding potential and the current amplitude for the glutamate-induced current (circles) and for the EJC (triangles). Current amplitudes obtained from three different preparations are plotted for each holding potential. The lines are linear regression lines for the glutamate-induced current (r^2=0.913, P<0.0001) and for the EJC (r^2=0.979, P<0.0001). The reversal potential for the glutamate-induced currents was +12.5 mV, and that for the EJCs was +14.9 mV.

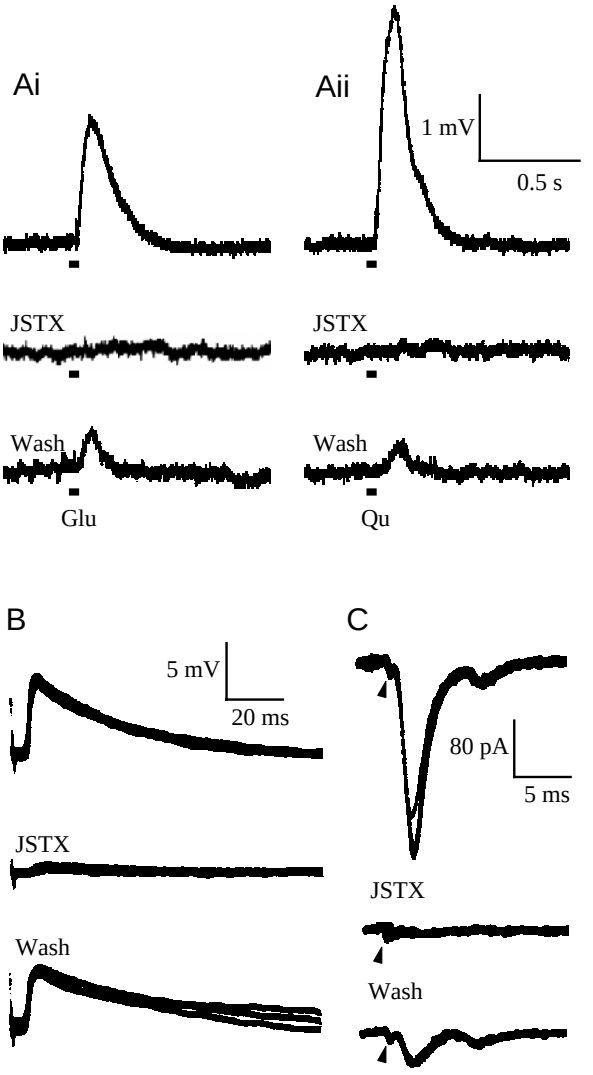

Fig. 9. Effects of Joro spider toxin (JSTX). (A) Effects of 10 μmol l^{-1} JSTX on the responses of the cardiac muscle of *Ligia exotica* to glutamate (Ai) and quisqualic acid (Aii). Glutamate (Glu, 1 mmol l^{-1}) or quisqualic acid (Qu, 1 mmol l^{-1}) was applied focally (indicated by bars below the recordings) to the same site of a quiescent heart in Ca^{2+}-free saline (control, top traces), 20 min after the onset of application of JSTX (middle traces) and 30 min after washout of JSTX (bottom traces). Muscle resting potential was −30 mV. (B) Effects of JSTX on excitatory junctional potentials (EJPs). Unitary EJPs were evoked by stimulation of a ganglionic nerve branch in normal saline (control, top trace), 15 min after the onset of application of JSTX (middle trace) and 30 min after washout of JSTX (bottom trace). Three sweeps are superimposed in each recording. Muscle resting potential was −47 mV. (C) Effects of JSTX on spontaneous excitatory junctional currents (EJCs). EJCs were recorded using a focal macropatch electrode in normal saline (control, top trace), 5 min after the onset of application of JSTX (middle) and 30 min after washout of JSTX (bottom trace). Note that JSTX has no effect on the action current of presynaptic impulses (arrowheads). Three sweeps are superimposed in each recording.

(Fig. 8Ai) and excitatory junctional currents (EJCs) (Fig. 8Aii) recorded at various holding potentials. The amplitude of both the glutamate-induced currents and the EJCs decreased as the holding potential became more depolarized. The relationships between the holding potential and the current amplitude were linear for both glutamate-induced currents (Fig. 8B, circles) and EJCs (Fig. 8B, triangles). The reversal potentials for the glutamate-induced current and for the EJC, estimated by extrapolation of the linear relationship were, +12.5 mV and +14.9 mV, respectively (Fig. 8B). The mean value of the reversal potential obtained from four preparations was +8.3±2.3 mV (mean ± S.E.M.) for the glutamate-induced current and +8.8±6.9 mV (mean ± S.E.M.) for the EJC (Student's *t*-test; no significant difference between values).

Effects of Joro spider toxin on glutamate responses and EJPs

Joro spider toxin (JSTX) is known to be a specific blocker of glutamatergic transmission (e.g. Kawai *et al.* 1982, 1983). The effects of JSTX (10 μmol l^{-1}) on the depolarizing membrane potential response of the cardiac muscle to focally applied glutamate (1 mmol l^{-1}) were examined in Ca^{2+}-free saline (*N*=8). Bath-application of JSTX for 20 min abolished the depolarizing response (Fig. 9Ai). No changes were observed in the membrane resistance of the cardiac muscle during the application of JSTX (data not shown). After washout of JSTX for 30 min, the amplitude of the depolarizing response recovered to approximately 30 % of the control value (Fig. 9Ai). The depolarizing membrane potential response of the cardiac muscle to quisqualic acid (1 mmol l^{-1}) was also blocked by JSTX (*N*=2) (Fig. 9Aii).

We also examined the effects of JSTX (10 μmol l^{-1}) on the EJPs evoked by ganglionic nerve stimulation in normal saline (*N*=28). Unitary EJPs were almost abolished by application of JSTX for 15 min. The EJPs recovered to approximately 25–75 % of the control amplitude after washout of JSTX for 30–60 min (Fig. 8B).

The effects of JSTX on the EJCs recorded using a macropatch electrode were also examined (*N*=5). The EJCs evoked by impulses generated spontaneously in the CG were abolished by application of JSTX (10 μmol l^{-1}) and recovered partially after washout of JSTX (Fig. 9C). No effects of JSTX on the action current of presynaptic impulses were observed (Fig. 9C, arrowheads).

Discussion

Innervation of the myocardium by CG neurones

All six CG neurones were labelled by intracellular injection of neurobiotin into any one of the six somata (Fig. 1B). This dye-coupling agrees with the observation of electrical connections among the CG neurones (Yamagishi and Ebara, 1985) and suggests that the endoplasm of the CG neurones is connected, probably *via* gap junctions. Tracer injection also revealed that all the CG neurones of *Ligia exotica* project their axons onto the myocardium, where they form nerve terminals (Figs 1, 2). The axon terminals bore many varicosities of various sizes (Figs 2C, 3C, 4Bi) that appeared similar to those on crustacean skeletal muscles (Atwood and Cooper, 1995, 1996; Cooper *et al.* 1996; Bradacs *et al.* 1997). It has been suggested that the varicosities of the axon terminals provide most of the synaptic input to the muscle fibres (Atwood and Cooper, 1995). Therefore, all six CG neurones appeared to be motoneurones innervating the myocardium. This indicates that the CG consists of six homogeneous neurones, each of which has pacemaker properties (Yamagishi and Ebara, 1985) and motor function. The CG of many decapods consists of nine neurones (e.g. Alexandrowicz, 1932), and these neurones are differentiated functionally into four small pacemakers and five large motoneurones (reviewed by Prosser, 1973; Wiens 1983; Cooke, 1988). In the CG of stomatopods, which consists of 15 neurones (Alexandrowicz, 1934), the pacemaker neurones are located anteriorly (reviewed by Prosser, 1973).

Polyneuronal innervation of cardiac muscle fibres by the CG neurones has been reported in several decapods (*Homarus americanus*, Anderson and Cooke, 1971; *Panulirus japonicus*, Kuramoto and Kuwasawa, 1980; *Portunus sanguinolentus*, Benson, 1981). In the heart of *Ligia exotica*, a nerve branch derived from the CG trunk contains the axons of several CG neurones (Fig. 3B) (Yamagishi and Ebara, 1985). Stimulation of the ganglionic nerve evoked compound EJPs in the cardiac muscle fibres (see Materials and methods). These observations suggest that the six CG neurones of *Ligia exotica* all innervate the myocardium and that the innervation sites overlap.

Glutamatergic transmission between the CG and myocardium

Several lines of evidence obtained in the present study lead us to conclude that the CG neurones of *Ligia exotica* are glutamatergic: (1) the glutamate-like immunoreactivity in the CG neurones; (2) the localization of the glutamate-sensitivity of the cardiac muscle to sites close to ganglionic nerve terminals; (3) the desensitization of glutamate and junctional receptors in response to glutamate; (4) the similar reversal potentials for glutamate-induced currents and EJCs; and (5) the blockage of glutamate-induced responses and EJPs by JSTX.

The neural system exhibiting glutamate-like immunoreactivity in the heart appeared to be consistent with the cardiac ganglion system stained by intracellular injection of neurobiotin (Figs 2, 3), suggesting that the CG neurones contain high concentrations of glutamate and that they make glutamatergic synapses onto the myocardium. In the giant marine isopod *Bathynomus doederleini*, the CG neurones are found to contain high concentration of glutamate (Yazawa *et al.* 1998) and exhibit glutamate-like immunoreactivity (Y. F-Tsukamoto and K. Kuwasawa, personal communication).

Focal application of glutamate revealed that the glutamate-sensitivity of the cardiac muscle was confined to sites close to nerve terminals bearing varicosities (Fig. 4B). The localization of glutamate sensitivity to sites close to the nerve terminals may reflect the localization of the glutamate receptors at junctional sites, as is observed in crustacean skeletal muscles (Takeuchi and Takeuchi, 1964; Onodera and Takeuchi, 1980). The

glutamate-sensitive sites were also highly sensitive to quisqualic acid (Fig. 4C), which is known to be a potent agonist of glutamate (e.g. Watkins and Evans, 1981). Moreover, the amplitude of the depolarizing responses of the cardiac muscle were dose-dependent (Fig. 5), and the depolarizing response was accompanied by a decrease in the membrane resistance (Fig. 6). These results suggest that glutamate is the most potent transmitter for the CG neurones of *Ligia exotica*.

Focally applied glutamate produced rapid desensitization of glutamate-sensitivity at the junctional sites of the cardiac muscle (Fig. 7A). Bath-applied glutamate decreased the amplitude of EJPs evoked by ganglionic nerve stimulation (Fig. 7B). These results suggest that exogenously applied glutamate causes desensitization of the junctional receptors at the neuromuscular junctions between the CG and myocardium. As reported for crustacean stomach muscle (Chiba and Tazaki, 1992; Tazaki and Tazaki, 1997), the desensitization of the junctional receptors by exogenously applied glutamate strongly supports the idea that the junctional receptor is a glutamate receptor (see Takeuchi and Takeuchi, 1972).

The estimated reversal potential for the EJC was similar to that for the glutamate-induced current (Fig. 8), suggesting that the junctional receptor at the neuromuscular junctions between the CG and myocardium is a glutamate receptor. In the myocardium of *Ligia exotica*, the reversal potentials of the EJC and the glutamate-induced current were approximately +8 mV, a value similar to those obtained in crustacean stomach muscles (Lingle and Auerbach, 1983; Chiba and Tazaki, 1992).

JSTX is known to be a specific blocker of glutamatergic transmission (Kawai *et al.* 1982, 1983; Abe *et al.* 1983). In the heart of *Ligia exotica*, JSTX blocked the glutamate-induced response and the EJP in the cardiac muscle (Fig. 9). It has been reported that JSTX irreversibly blocks the glutamate potential and the EJP in crustacean skeletal muscle (Kawai *et al.* 1982; Abe *et al.* 1983) and stomach muscle (Chiba and Tazaki, 1992). However, in the present study, both the glutamate potential and the EJP recovered partially after washout of JSTX (Fig. 9). This suggests that there may be differences in the mechanism of action of JSTX at the neuromuscular junctions in the heart of *Ligia exotica* from that at other known glutamatergic synapses.

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