

TRANSFER OF THE HEART PACEMAKER DURING JUVENILE DEVELOPMENT IN THE ISOPOD CRUSTACEAN *LIGIA EXOTICA*

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Accepted 11 July 1997

Summary

Developmental changes in heartbeat pacemaker mechanisms were examined electrophysiologically in the isopod crustacean *Ligia exotica*. The heartbeat of embryos and early juveniles was myogenic. The heart muscle cells were coupled electrically, and no localized pacemaker activity was found in the heart. In newly hatched juveniles, the cardiac ganglion exhibited no spontaneous activity, although stimulation of the cardiac ganglion produced excitatory junctional potentials (EJPs) in the heart muscle. The myogenic activity of the heart was reset and entrained by the EJPs evoked by ganglionic stimulation. During juvenile development, spontaneous EJPs appeared irregularly in the heart muscle. Later in development, the cardiac ganglion started rhythmic bursting, and each

muscle response followed a ganglionic burst discharge and overlapped the EJPs evoked by ganglionic activity. At this point, the activity of the cardiac ganglion was suppressed by application of tetrodotoxin (TTX); however, even in old adults, both muscle activity and the heartbeat continued following TTX application. Heartbeat frequency was lower in TTX-containing saline than in normal saline. These results show that, during juvenile development, the heart pacemaker is transferred from the heart muscle to the cardiac ganglion, which becomes the primary pacemaker and entrains the heart muscle activity to a higher frequency via EJPs.

Key words: *Ligia exotica*, Crustacea, development, heart, pacemaker.

Introduction

The hearts of many crustaceans and the chelicerate *Limulus polyphemus* are known to be neurogenic (reviewed by Krijgsman, 1952; Maynard, 1960; Prosser, 1973). In these animals, the heart muscle has no inherent automaticity and the cardiac ganglion situated in the heart acts as a pacemaker to drive the heart muscle via excitatory neuromuscular junctional potentials (EJPs). Therefore, the heart stops beating if the cardiac ganglion is removed (reviewed by Krijgsman, 1952; Maynard, 1960) or following application of tetrodotoxin (TTX), which suppresses neuronal spiking in the cardiac ganglion (Rulon *et al.* 1971; Anderson and Cooke, 1971).

Carlson (1904a) first established the neurogenic nature of the heartbeat in adult *Limulus polyphemus*. Carlson and Meek (1908) reported from histological observations that the embryonic heart of *Limulus polyphemus* was not innervated by the cardiac ganglion for approximately 10 days after the onset of beating, and concluded that the embryonic heartbeat was myogenic. This conclusion was supported by reports of differing activation energy between embryonic and adult hearts (Crozier and Stier, 1927) and of developmental changes in the effects of acetylcholine (ACh) on embryonic hearts (Prosser,

1942). However, Gibson and Lang (1979), using both electron microscopic and electrophysiological methods, could find no evidence that the embryonic *Limulus polyphemus* heart passes through a myogenic phase and concluded that the heartbeat is neurally driven throughout development.

The neurogenic nature of the crustacean heartbeat was first reported by Alexandrowicz (1931) in adults of the isopod *Ligia oceanica* as a result of sectioning the cardiac ganglion. The cardiac ganglion of *Ligia* (*L. oceanica* and *L. exotica*) is composed of six neurones (Suzuki, 1934; Alexandrowicz, 1952; Yamagishi and Ebara, 1985). In the adult, the cardiac ganglion exhibits rhythmic bursting and each heartbeat follows a ganglionic burst discharge (Ai, 1966; Yamagishi and Ebara, 1985). However, Ai (1966) recorded spontaneous activity of the heart muscle in addition to neurogenic activity and concluded that the heart of *Megaligia exotica* (*Ligia exotica*) adults has both neurogenic and myogenic properties.

Recently, Yamagishi (1996) showed that muscle activity and beating persist in the heart of *Ligia exotica* early juveniles in TTX-containing saline and that the rhythm of the muscle activity is reset and changed in frequency by current injection

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into the heart muscle. Yamagishi (1996) therefore concluded that the heartbeat is myogenic, suggesting that the heart pacemaker of *Ligia exotica* is transferred from the heart muscle to the cardiac ganglion during development. The aims of the present study were to determine (1) the time course of embryonic and juvenile development, (2) the mechanism of the myogenic heartbeat, (3) the developmental stage at which the cardiac ganglion becomes the heart pacemaker and (4) the mechanism of the neurogenic heartbeat. The results show that, during juvenile development, the heart pacemaker is transferred from the heart muscle to the cardiac ganglion, allowing a switch from myogenic to neurogenic control. The type of neurogenic heartbeat is different from that known previously in any other crustacean. Some of the results presented here have appeared previously in abstract form (Yamagishi, 1990, 1995).

Materials and methods

Animal collection and maintenance

Over 500 specimens of various developmental stages of the littoral isopod *Ligia exotica* (Roux) were used. Adult males and females, 20–40 mm in body length, were collected on the Pacific coast at Shimoda and Choshi, Japan. They were kept in a plastic tank containing a small amount of artificial sea water (Senju Seiyaku) and a cement block, and were fed on dried green algae and dried fish. The colony was maintained in the laboratory during the breeding period (April to September). Females that held eggs were kept individually in small plastic containers to obtain embryos and juveniles of known stages. Developmental stages of embryos and juveniles were determined and expressed as the number of days before and after hatching.

Preparations and electrical recordings

The method of dissection, the composition of the physiological saline solution and the anatomy of the adult heart were as described previously (Yamagishi and Ebara, 1985; Yamagishi *et al.* 1989).

The heart (less than 1 mm in length and 0.1 mm wide in late embryos) was kept intact in the pericardial cavity and was isolated together with the dorsal carapace. It was pinned ventral-side up in the experimental chamber. The chamber was perfused continuously with aerated normal physiological saline having the following composition (in mmol l⁻¹): NaCl 586, KCl 14, CaCl₂ 25, MgCl₂ 16.5, MgSO₄ 4.5, Tris-HCl 5 (pH 7.4). In some experiments, 10⁻⁶ mol l⁻¹ tetrodotoxin (TTX) (Sigma) was added to the saline. Intracellular activity of the heart muscle cells was recorded using a conventional glass microelectrode filled with 3 mol l⁻¹ KCl. For current injection, a second microelectrode was inserted into the heart muscle at a distance from the recording electrode. A glass suction electrode was used to record the ganglionic activity from the trunk portion of the cardiac ganglion or for stimulating the trunk. In these cases, the ventral heart wall was excised at the central region of the heart to expose the cardiac ganglion

situated on the dorsal heart wall inside the heart. In the heart of adults, ganglionic activity was also recorded from the nerve branches of the cardiac ganglion. To achieve this, the anterior or posterior nerve branch of the cardiac ganglion was exposed by partial removal of the heart wall and cut at the peripheral side. The proximal cut end of the nerve was then sucked into the suction electrode. Contraction of the heart (mechanogram of the heartbeat) was recorded in a preparation pinned dorsal-side-up in the chamber. Part of the dorsal carapace was removed over the middle region of the heart. The suspensory ligament, which was left attached to the heart, was tied using fine thread and was connected to the mechano-electric transducer (Yamagishi *et al.* 1989). Data were recorded using an FM tape recorder and a chart recorder. All experiments were carried out at a temperature of 20–23 °C.

Light microscopy and electron microscopy

The hearts of both newly hatched juveniles and adults were used for light microscopy and electron microscopy. The juvenile heart was isolated intact in the pericardial cavity together with the dorsal carapace. In adults, only the heart was isolated. These hearts were prefixed using 2.5% glutaraldehyde and postfixed using 1% osmium tetroxide. They were then dehydrated using an ethanol series and embedded in modified Spurr's resin (Kushida, 1980). Thick sections (1 µm) were stained with Toluidine Blue for light microscopy. Thin sections (0.1 µm) were stained with uranyl acetate and lead citrate for electron microscopy. For further details of the methods used, see Yamagishi and Hirose (1992).

Results

Embryonic and juvenile development

After copulation, the female moults and holds 80–120 fertilized eggs in the brood pouch situated on the ventral surface of the abdomen. Fig. 1 shows the time course of embryonic and juvenile development of *Ligia exotica*. The embryo develops in the egg for approximately 3 weeks before hatching as a juvenile. The heartbeat starts at approximately 2 weeks of embryonic development. The newly hatched juvenile is approximately 3 mm in body length and has six pairs of legs. Several days after hatching, the juveniles are released from the mother's brood pouch. The juvenile stage lasts for approximately 3 weeks, during which time the juvenile moults twice before becoming an immature adult. It is then approximately 5 mm in body length and has seven pairs of legs. The immature adult takes 4–5 months to mature (approximately 20 mm in body length) and 3–4 years to reach maximum body length (over 40 mm).

Anatomy of newly hatched juvenile hearts

In adult *Ligia exotica*, the heart is tubular, is located in the dorsal side of the posterior half of the body and is suspended in the pericardial cavity by suspensory ligaments. The heart wall consists of a single layer of muscle fibres forming a right-

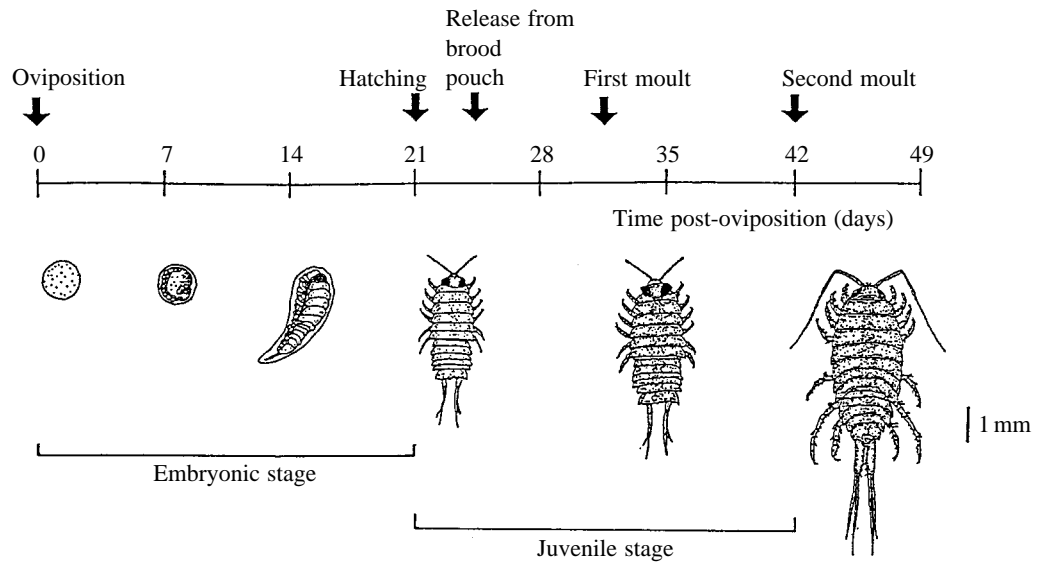


Fig. 1. The time course of embryonic and juvenile development of *Ligia exotica*. See text for further details.

handed spiral. The cardiac ganglionic trunk, which contains six neurones, runs longitudinally in the midline of the inner surface of the dorsal heart wall. Fig. 2A–C shows morphological features of the heart of newly hatched juveniles. At this stage of development, the heart tube was approximately 1 mm in length and 0.1 mm wide and was suspended in the pericardial cavity as in the adult (Fig. 2A). In electron micrographs, the heart wall could be seen clearly to consist of a single layer of striated muscle (Fig. 2B). Many axons were found running through the trunk portion of the cardiac ganglion in both the juvenile (Fig. 2C) and adult (Fig. 2D) heart. These observations confirm that the cardiac ganglion is present in the heart of newly hatched juveniles.

Myogenic activity in the heart of embryos and early juveniles

The heart in late embryos and early juveniles beats at a frequency of 200–400 min^{-1} . Intracellular activity of the heart muscle and impulse activity of the cardiac ganglion were recorded simultaneously. As shown in Fig. 3A, each heartbeat was associated with a rhythmic burst discharge of the heart muscle (upper trace). No impulse activity corresponding to the muscle activity was recorded extracellularly from the trunk portion of the cardiac ganglion (Fig. 3A, lower trace).

Muscle activity in the heart of early juveniles is composed of oscillatory slow potentials with superimposed TTX-sensitive spike potentials; the heartbeat and muscle activity lacking the spike potentials both persist in TTX-containing saline (Yamagishi, 1996). The effects of TTX on the heartbeat were further examined in the heart of embryos and early juveniles. Perfusion of saline containing $10^{-6} \text{ mol l}^{-1}$ TTX, which suppresses neuronal spiking in the cardiac ganglion (see below), rapidly eliminated spike potentials in the heart muscle while the slow oscillatory potentials persisted (Fig. 3B, upper trace). The frequency of the slow oscillatory potentials in TTX-containing saline was similar to that of muscle activity in normal saline (Fig. 3B, lower trace). Myogenic activity of the heart was thus confirmed in embryos and early juveniles.

Diffuse myogenicity

Intracellular muscle activity was recorded simultaneously at two different sites in the heart in both embryos and early juveniles. As shown in Fig. 4A, burst discharges of the muscle cells appeared almost simultaneously in both recording sites with no obvious differences in amplitude. Slight differences in the timing of the bursts of a few milliseconds were often observed, but these did not show a constant phase relationship during measurements.

The intracellular activity of muscle fragments isolated from different regions of the heart was also examined. As shown in Fig. 4B, fragments isolated from different regions of the heart exhibited rhythmic activity similar to that recorded in the intact heart. These results suggest that the heart has a diffuse myogenic nature.

Electrical coupling of heart muscle cells

To examine electrical coupling of the muscle cells in the heart, a brief hyperpolarizing current pulse was injected repetitively into the muscle at various sites within the heart. Regardless of the site of injection, electrotonic potentials were recorded intracellularly from muscle cells in all regions of the heart (Fig. 5A). The potentials recorded tended to be smaller with increasing distance from the stimulation site.

The presence of electrical pathways among the muscle cells suggests that synchronization of muscle activity in the heart is achieved through electrical connections between the muscle cells. To investigate this possibility, the effects on the heartbeat of cutting the cardiac ganglionic trunk were examined (Fig. 5B). Muscle activity was recorded simultaneously from anterior (upper trace) and posterior (lower trace) regions of the heart. Immediately after cutting the cardiac ganglionic trunk in the central region of the heart, muscle activity in the anterior and posterior regions was irregular and was not synchronized (Fig. 5Bi). Activity in one region caused changes in the electrotonic membrane potential in the other regions (Fig. 5Bi, arrowheads). EJP-like potentials, probably caused by damage

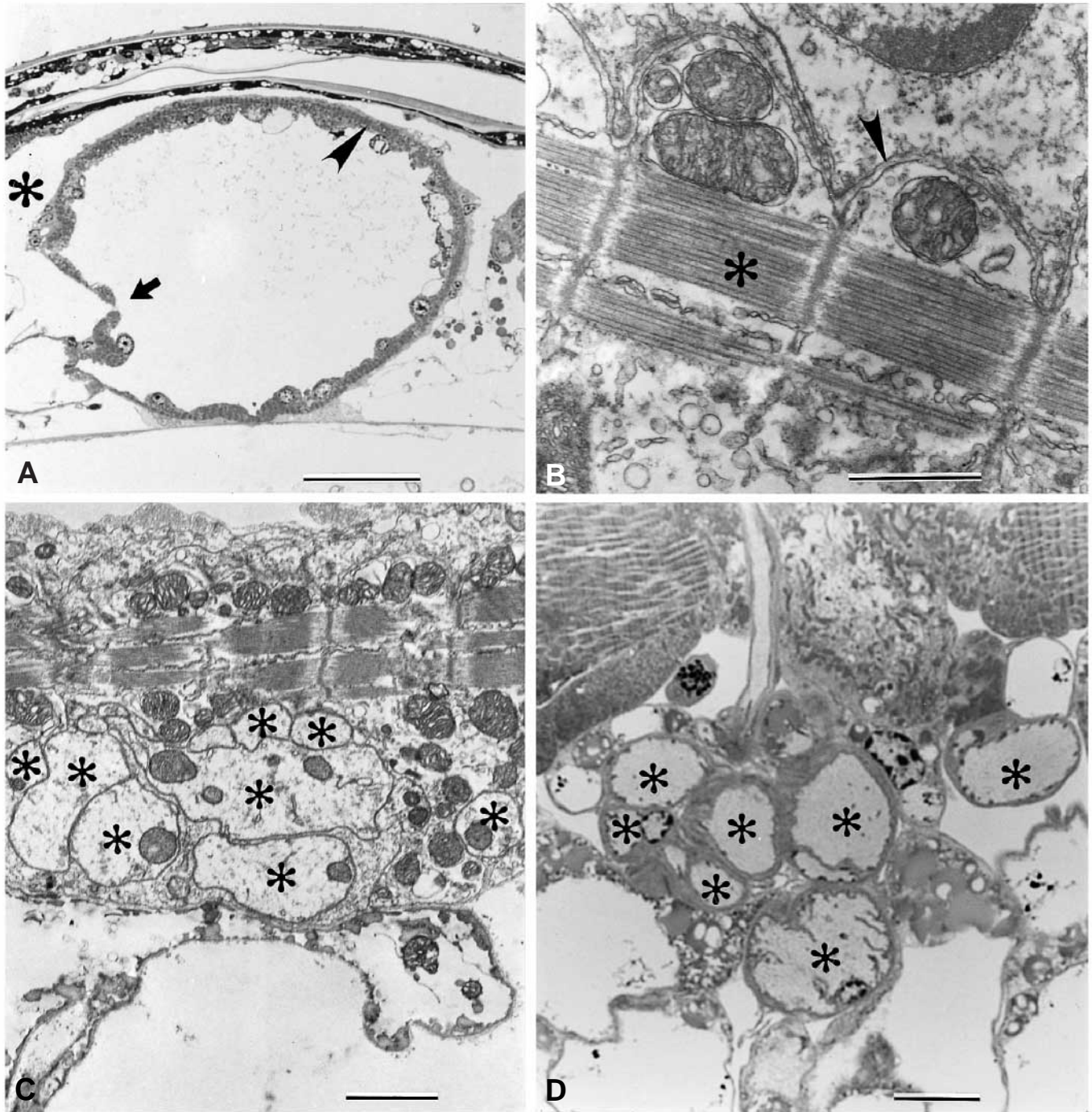


Fig. 2. Light micrographs (A,D) and electron micrographs (B,C) of early juvenile and adult *Ligia exotica* hearts. (A,B,C) Juveniles just after hatching, (D) an adult 34 mm in body length. (A) Cross section of the heart and the surrounding tissues through the central region of the heart. The heart tube (arrowhead) is suspended in the pericardial cavity (asterisk). The arrow indicates the ostium. (B) Cross section of the heart wall. The heart wall consists of an adventitia (arrowhead) and a single layer of striated muscle (asterisk). (C,D) Cross sections of the cardiac ganglionic trunk. The asterisks indicate the axons. Scale bars, 50 μ m (A), 1 μ m (B), 2 μ m (C) and 10 μ m (D).

to the cardiac ganglion, were usually observed for several minutes after the cut was made (Fig. 5Bi, asterisks). Synchronization of the activity between the two regions of the heart was gradually achieved with time, seemingly mediated by the electrotonic potentials (Fig. 5Bii,iii).

Effects of current injection on myogenic activity

In the heart of early juveniles, current injection into the heart muscle causes a change in frequency and resets the rhythm of the muscle activity persisting in TTX-containing saline (Yamagishi, 1966). The effects of current injection into the

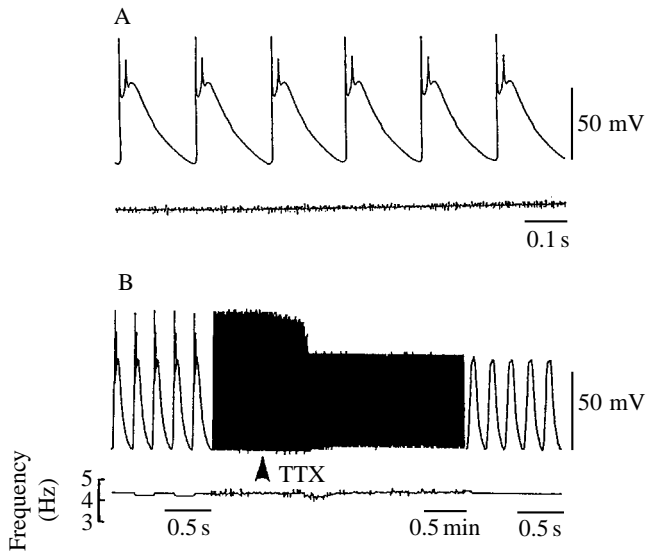


Fig. 3. Muscle activity in early juvenile hearts. (A) Simultaneous recordings from the heart muscle and cardiac ganglion. Upper trace, activity recorded intracellularly from the heart muscle. Lower trace, activity recorded extracellularly from the cardiac ganglionic trunk. From a juvenile 1 day after hatching. (B) Effects of tetrodotoxin (TTX) on muscle activity. Upper trace, heart muscle activity recorded intracellularly. Lower trace, frequency of muscle burst discharge (Hz). The arrowhead shows the time when the perfusion saline was changed to TTX-containing saline ($10^{-6} \text{ mol l}^{-1}$). From a juvenile 2 days after hatching. Note the different time scale for the central portion of the trace.

heart muscle on its activity in normal saline were also examined in embryos and early juveniles. As shown in Fig. 6A, application of a brief depolarizing current pulse evoked a burst discharge at any phase within the interburst

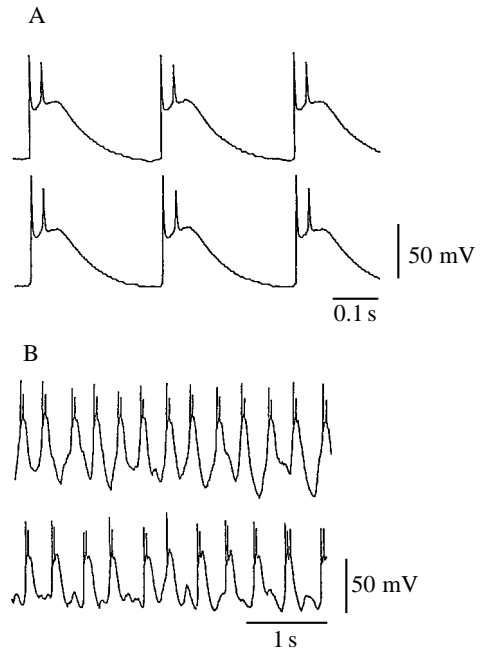
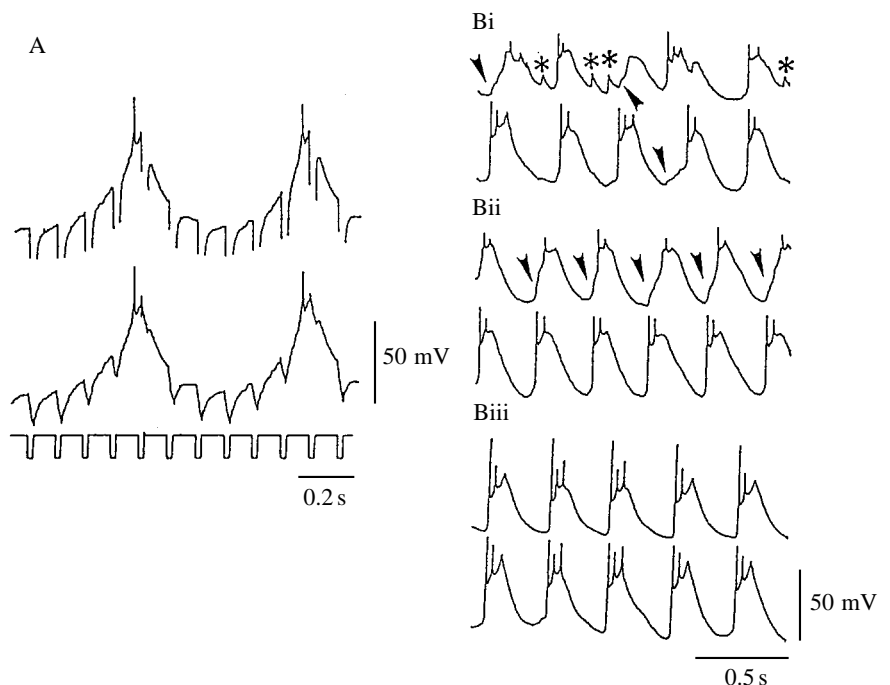


Fig. 4. Diffuse myogenicity in early juvenile hearts. (A) Intracellular muscle activity recorded simultaneously from two different sites, near the anterior (upper trace) and posterior (lower trace) end of the heart. From a juvenile 2 days after hatching. (B) Intracellular muscle activity recorded from the heart cut transversely into six separate parts. Records obtained simultaneously from the first (from the anterior end of the heart, upper trace) and the fifth (near the posterior end of the heart, lower trace) fragments are shown. From a juvenile 1 day after hatching.

interval (the interval between the first spikes of adjacent bursts) and reset the rhythm of the subsequent muscle activity. If a train of brief pulses was applied, the frequency of muscle activity could be entrained to a higher frequency than that

Fig. 5. Electrical coupling among the heart muscle cells. (A) Intracellular muscle activity recorded simultaneously from two different sites, near the anterior (top trace) and posterior (middle trace) end of the heart. Brief hyperpolarizing current (20 ms, 10 nA) was applied at 10 Hz (bottom trace) via an electrode inserted into the anterior portion through a bridge circuit. From a juvenile 1 day after hatching. (B) Synchronized muscle activity recorded after cutting the cardiac ganglionic trunk in the central region of the heart. Intracellular muscle activity was recorded simultaneously from two different sites, near the anterior (upper trace) and the posterior (lower trace) end of the heart. Records obtained 2 min (i), 10 min (ii) and 20 min (iii) after the cut were shown. The asterisks show the EJP-like potentials. The arrowheads show the electrotonic potentials that correspond to the activity in the other part of the heart. From a juvenile 2 days after hatching.



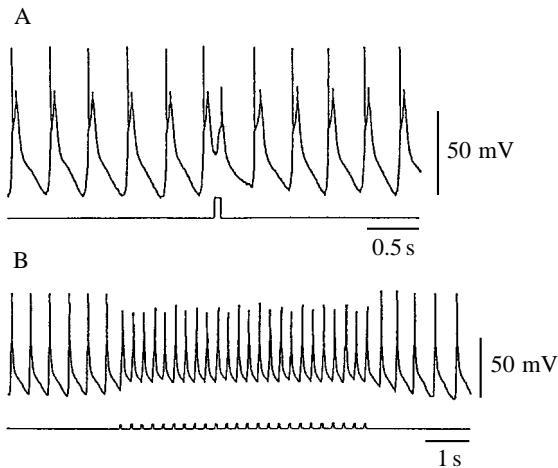
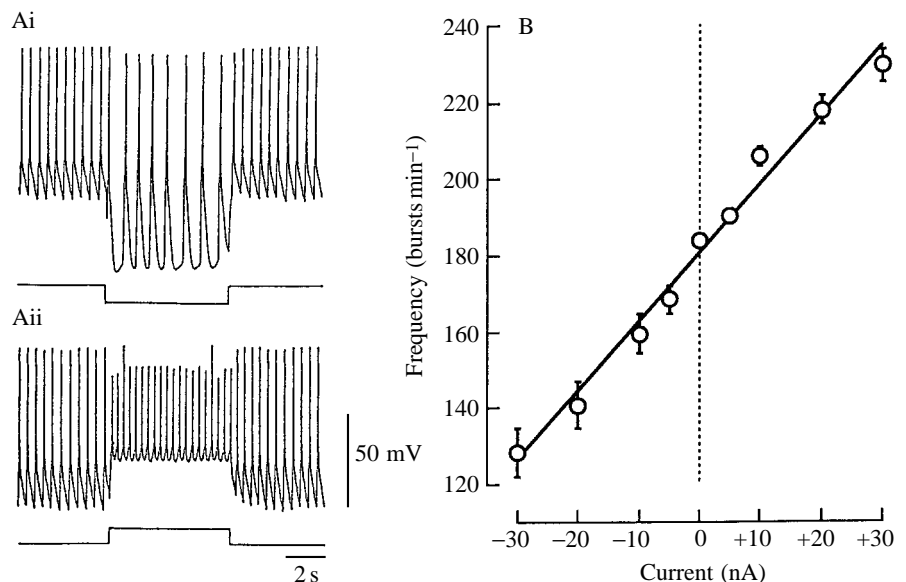


Fig. 6. Effects of current injection on muscle activity in the heart. Intracellular muscle activity was recorded from a site near the anterior end (upper trace) of the heart, and current pulses were injected into the heart muscle at a site near the posterior end (lower trace) of the heart. (A) Resetting of the muscle rhythm. A brief depolarizing current (50 ms, 10 nA) was injected. (B) Entrainment of the muscle rhythm. A brief depolarizing current (50 ms, 10 nA) was injected at a frequency of 4 Hz. A and B are from the same embryo 1 day before hatching.

recorded in the absence of stimulation (Fig. 6B). These effects occurred regardless of the site of injection.

The effects of long-duration hyperpolarizing and depolarizing current pulses on the heartbeat were also examined. The frequency of the muscle activity decreased during hyperpolarization and increased during depolarization (Fig. 7A). Fig. 7B shows the relationship obtained from a single preparation between the frequency of muscle activity and the amplitude of the injected current. The frequency of the muscle activity increased with increasing intensity of depolarizing current and decreased with decreasing intensity of hyperpolarizing current.

Fig. 7. Dependence of membrane potential upon muscle activity. (A) Effects of longer-duration current injection on the muscle rhythm. Upper trace, muscle activity recorded intracellularly. Lower trace, injected current. Hyperpolarizing (i) or depolarizing (ii) current (10 nA) was injected into the heart muscle. From an embryo 1 day before hatching. (B) Relationship between frequency of muscle activity and injected current amplitude. Values are means \pm S.E.M. of instantaneous frequency of 10 successive bursts during application of hyperpolarizing (-) or depolarizing (+) current. The dashed line shows the frequency under unstimulated conditions. The equation for the regression line is $y=180.656+1.809x$, $r^2=0.970$, $P<0.0001$. From a juvenile 1 day after hatching.



Innervation of heart muscle by the cardiac ganglion in newly hatched juveniles

To investigate possible cardiac ganglionic innervation of the heart muscle in newly hatched juveniles, the effects of stimulation of the cardiac ganglion on heart muscle activity were examined. A single brief (0.5 ms) electric pulse was applied to the central portion of the cardiac ganglionic trunk at various phases of the interburst interval of the muscle activity. The interburst interval is defined above, and the stimulation phase was taken as the time from the peak of the first spike of a burst to the time of the applied stimulus expressed as a percentage of the preceding control interburst interval. When the stimulus was applied at an early phase of the interburst interval (Fig. 8A), it evoked an EJP-like small depolarizing potential in the heart muscle. The amplitude of the response was larger when the stimulus was applied to a more hyperpolarized membrane potential (Fig. 8B). When the stimulus was applied at a phase later than approximately 60–70%, it induced an action potential in the heart muscle, and the rhythm of the subsequent muscle activity was shifted to an earlier phase (Fig. 8C). These results suggest that the cardiac ganglion innervates the heart muscle in newly hatched juveniles and can reset the muscle rhythm *via* EJPs.

The effects of repetitive ganglionic stimulation on the myogenic activity of the heart were examined in newly hatched juveniles (Fig. 9). The frequency of the unstimulated muscle activity was approximately 4 Hz. The frequency of the muscle activity was entrained to stimulus frequencies of 5 (Fig. 9A) and 6 Hz (Fig. 9B), but could not be entrained to a stimulus frequency of 7 Hz (Fig. 9C). The cardiac ganglion can therefore entrain the muscle rhythm to a higher frequency within a limited frequency range.

Appearance of spontaneous EJPs

In juveniles 5–12 days after hatching, spontaneous EJP-like small depolarizing potentials appeared superimposed

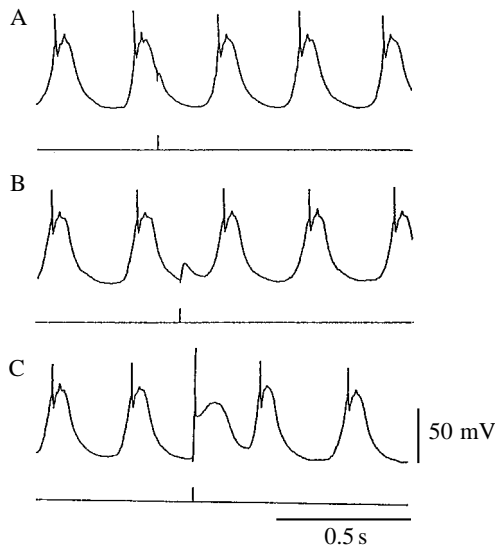


Fig. 8. Effects of cardiac ganglionic stimulation on heart muscle activity in newly hatched juveniles. Upper trace, muscle activity recorded intracellularly. Lower trace, stimulus applied. A single brief pulse (duration 0.5 ms) was applied to the cardiac ganglionic trunk at various phases of the muscle activity. The stimulation phase (as a percentage interburst interval) was 30% (A), 53% (B) and 78% (C), respectively. From a juvenile just after hatching.

irregularly on the heart muscle activity (12 examples in 78 preparations). EJP-like potentials were recorded synchronously from both central and posterior regions of the heart, with the potential recorded from the posterior portion being larger in amplitude than that from the central portion

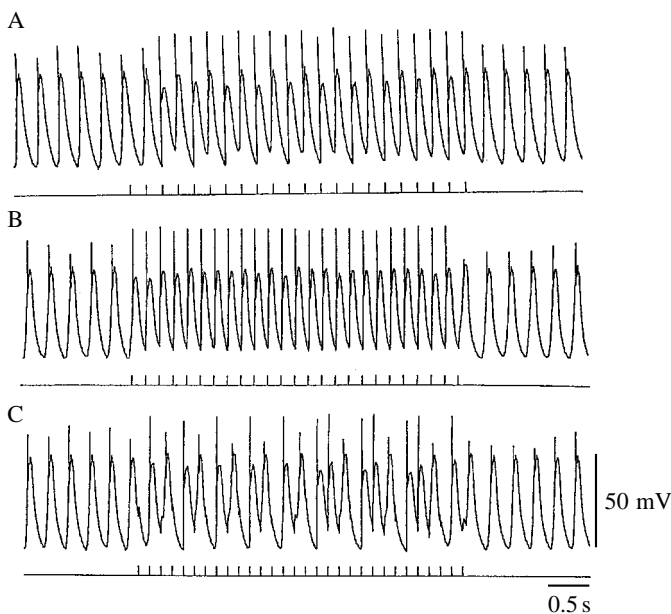


Fig. 9. Entrainment of heart muscle activity by cardiac ganglionic stimulation. Upper trace, muscle activity recorded intracellularly. Lower trace, stimuli applied. A brief pulse (duration 0.5 ms) was applied at stimulation frequencies of 5 Hz (A), 6 Hz (B) and 7 Hz (C) to the cardiac ganglionic trunk. From a juvenile just after hatching.

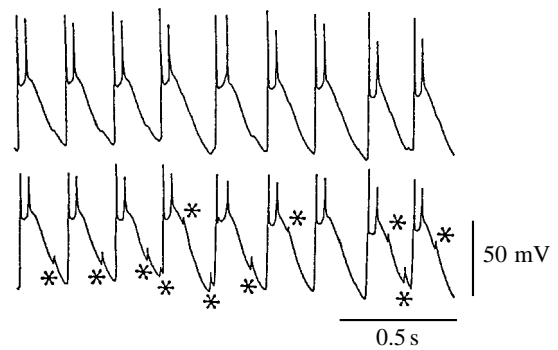


Fig. 10. Spontaneous EJP-like small depolarizing potentials. Intracellular activity of the heart muscle was recorded simultaneously from central (upper trace) and posterior (lower trace) regions of the heart. The asterisks indicate the EJP-like potentials. From a juvenile 8 days after hatching.

(Fig. 10, asterisks). This amplitude difference was larger with increasing distance between the two recording sites. These results suggest that the EJP-like potentials are generated in a localized region of the heart.

The effects of current injection into the heart muscle on the EJP-like potentials are shown in Fig. 11 in an example in which the EJP-like potentials appeared as bursts. Under unstimulated conditions (Fig. 11A), the frequency of the spontaneous activity of the muscle was $153 \pm 2.1 \text{ min}^{-1}$ (mean \pm s.d.). During application of hyperpolarizing direct current (Fig. 11B), the frequency of muscle activity decreased by 34% (to 101 min^{-1}), while the number of bursts of EJP-like potentials was unchanged (63 min^{-1}). This suggests that the EJP-like potentials are not generated endogenously in the heart muscle.

To test whether the EJP-like potentials were evoked neurally, the effects of TTX on the EJP-like potentials were investigated. TTX suppresses impulse generation in the cardiac ganglion (see

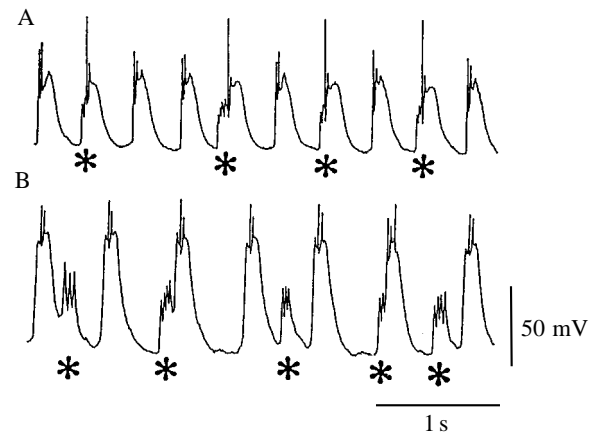


Fig. 11. Effects of current injection on EJP-like potentials in heart muscle. (A) Spontaneous activity recorded intracellularly from the heart muscle. (B) Activity during injection of hyperpolarizing current (40 nA) into the heart muscle *via* a second microelectrode. Asterisks under both records indicate EJP-like potential bursts. From a juvenile 10 days after hatching.

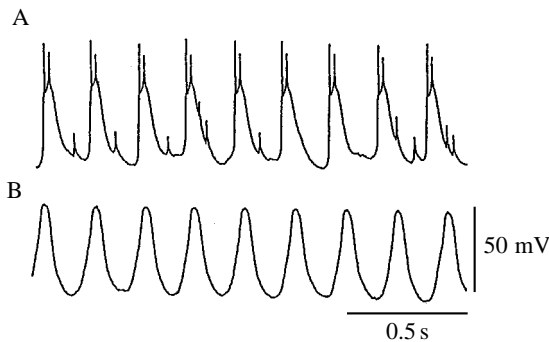


Fig. 12. Effects of tetrodotoxin (TTX) on EJP-like potentials. (A) Intracellular activity recorded from the heart muscle. (B) Activity recorded 1 min after changing the perfusion medium to $10^{-6} \text{ mol l}^{-1}$ TTX-containing saline. EJP-like potentials were abolished following TTX application. From a juvenile 12 days after hatching.

below). The EJP-like potentials, which were superimposed irregularly on the muscle spontaneous activity (Fig. 12A), were completely eliminated by application of $10^{-6} \text{ mol l}^{-1}$ TTX (Fig. 12B). The effect was reversible, and the EJP-like potentials reappeared after washout of TTX (results not shown). These results suggest that the EJP-like potentials are EJPs evoked by the spontaneous activity of the cardiac ganglion.

Spontaneous periodic burst discharges of the cardiac ganglion

The cardiac ganglion began to show spontaneous periodic bursting during juvenile development. The earliest periodic burst discharges were recorded extracellularly from the cardiac ganglionic trunk of a juvenile 9 days after hatching. As shown in Fig. 13A, each muscle response followed a burst discharge in the cardiac ganglion. EJPs were not usually detectable in the rising phase of muscle activity.

To detect the EJP underlying the muscle activity, the heart wall was pinched using fine tweezers to cause a deterioration in muscle activity, but taking care to keep the cardiac ganglion intact. In some cases, 'notches' representing the EJP component, were observed clearly in the rising phase of the muscle activity (Fig. 13Bi). During subsequent injection of hyperpolarizing direct current into the muscle, the muscle potentials were largely eliminated and only periodic EJP bursts were recorded (Fig. 13Bii). The frequency of EJP bursts was unchanged or decreased slightly during hyperpolarization. These results suggest that the muscle activity was entrained by the activity of the cardiac ganglion *via* EJPs.

Effects of TTX on adult heartbeats

After the cardiac ganglion began periodic bursting during juvenile development, heartbeats were associated with ganglionic burst discharges. The effects of TTX on the cardiac ganglionic and muscle activities were then examined in adult hearts. The intracellular activity of the heart muscle and impulse activity of the cardiac ganglion were recorded simultaneously (Fig. 14A). Each muscle response followed a burst discharge of the cardiac ganglion. During application of $10^{-6} \text{ mol l}^{-1}$ TTX,

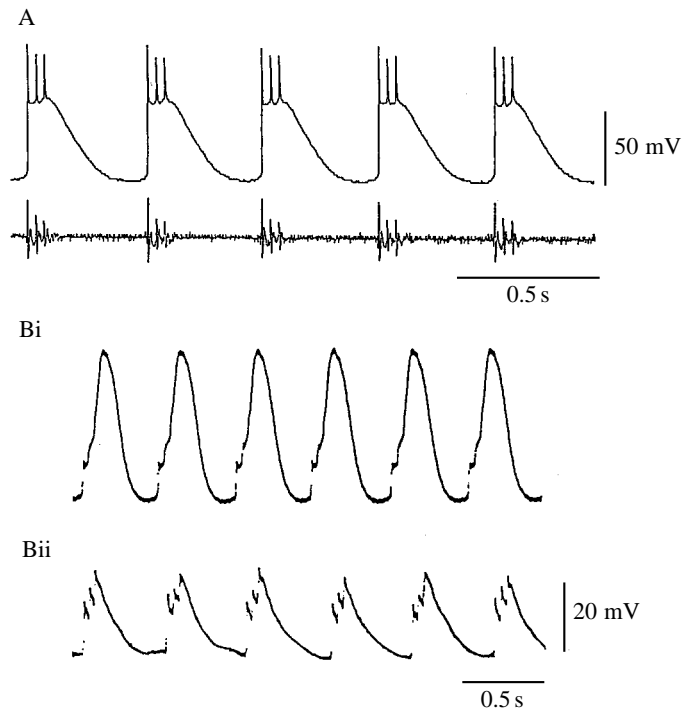


Fig. 13. Periodic burst discharges of the cardiac ganglion and EJPs underlying the muscle activity in late juvenile hearts. (A) Simultaneous recording of intracellular activity of the heart muscle (upper trace) and impulse activity of the cardiac ganglionic trunk (lower trace). From a juvenile 17 days after hatching. (B) Intracellular activity recorded from the heart muscle (upper trace). The heart wall was pinched, causing deterioration in muscle activity. The muscle membrane was then hyperpolarized by current injection (30 nA) into the heart muscle (lower trace). From a juvenile 16 days after hatching.

the ganglionic burst discharges were eliminated rapidly, while the heart muscle exhibited oscillatory slow potentials. The frequency of the oscillatory slow potential reached a constant level during perfusion of TTX-containing saline that was lower than that recorded in normal saline (Fig. 14B). Transient changes in the frequency of the muscle activity were usually observed immediately following application and washout of TTX. These results suggest that the heart muscle of adults also possessed endogenous oscillatory properties.

To confirm the presence of myogenicity in adult hearts, the effects of current injection into the heart muscle on the muscle activity were examined in normal and TTX-containing salines (Fig. 15). The frequency of muscle activity in normal saline was 3.7 Hz (Fig. 15Ai), and it was not affected by hyperpolarizing current injection into the heart muscle (Fig. 15Aii). In contrast, in saline containing $10^{-6} \text{ mol l}^{-1}$ TTX, the frequency of the muscle slow oscillatory potential was 3.7 Hz, and it decreased to 3.1 Hz (16% reduction) following injection of hyperpolarizing current (Fig. 15B). These results strongly support the suggestion that the muscle activity recorded in TTX-containing saline is endogenous.

To investigate the effects of TTX on the adult heartbeat, the impulse activity of the cardiac ganglion and a mechanogram

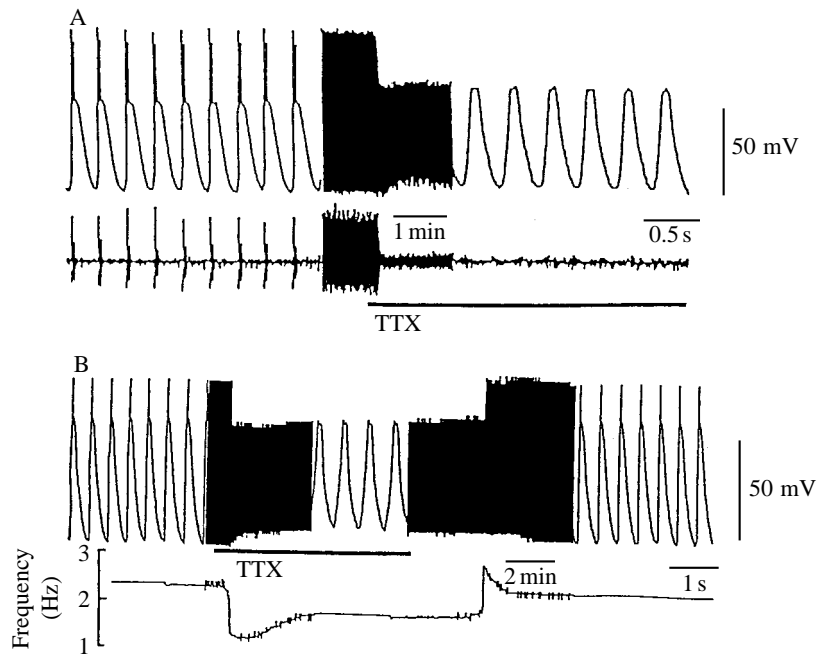


Fig. 14. Effects of tetrodotoxin (TTX) on cardiac ganglionic and muscle activities in adult hearts. (A) Simultaneous recording of intracellular activity of the heart muscle (upper trace) and impulse activity of the cardiac ganglionic trunk (lower trace). $10^{-6} \text{ mol l}^{-1}$ TTX was applied during the period indicated by the black bar. From an adult 22 mm in body length. (B) Muscle activity recorded intracellularly (upper trace) and the frequency of muscle activity (lower trace). $10^{-6} \text{ mol l}^{-1}$ TTX was applied during the period indicated by the black bar. From an adult 26 mm in body length. Note the changes in time scale.

of the heartbeat were recorded simultaneously (Fig. 16). In normal saline, each heartbeat followed a burst discharge of the cardiac ganglion at a frequency of 1.9 Hz. During application of $10^{-6} \text{ mol l}^{-1}$ TTX, the heart continued to beat at 1.5 Hz in the absence of cardiac ganglionic activity. Following washout of TTX, cardiac ganglionic activity recovered and the frequency of the heartbeat returned to 1.9 Hz. Thus, the heart of adults continued to beat following application of TTX, although the beat frequency in normal saline was higher than that in TTX-containing saline.

Discussion

Myogenic heartbeats

The heartbeats of late embryos and early juveniles of *Ligia exotica* were associated with periodic burst discharges of the heart muscle in the absence of cardiac ganglion activity (Fig. 3A). Moreover, the heart continued to beat in saline containing TTX, which suppresses impulse generation in the cardiac ganglion of *Ligia exotica*, as well as in other crustacean cardiac ganglia (Tazaki, 1971; Tazaki and Cooke, 1979). Heart muscle activity of *Ligia exotica* early juveniles consists of oscillatory slow potentials with superimposed TTX-sensitive spike potentials (Yamagishi, 1996). The heart continued to beat and the oscillatory slow potential was maintained in TTX-containing saline (Fig. 3B), indicating that the heartbeats of embryos and early juveniles of *Ligia exotica* are myogenic.

The heartbeat of *Limulus polyphemus* and many crustaceans originates in the cardiac ganglion, which produces periodic burst discharges (reviewed by Krijgsman, 1952; Maynard, 1960). These ganglionic burst discharges evoke EJPs on the heart muscle and cause neurogenic heartbeats (Abbott *et al.* 1969; Parnas *et al.* 1969; Van der Kloot, 1970; Anderson and Cooke, 1971; Kuramoto and Kuwasawa, 1980). Following

application of TTX, neuronal spiking in the cardiac ganglion is suppressed and the heartbeat stops completely (Anderson and Cooke, 1971; Watson *et al.* 1983). Myogenic activity can be induced in deganglionated *Limulus polyphemus* hearts by application of modified physiological solutions (reviewed by Krijgsman, 1952), by stretch (Lang, 1971) or by application of a peptide, proctolin (Watson *et al.* 1983). However, myogenic activity was not found under normal physiological conditions (Carlson, 1904b). In crustaceans, it has been suggested on the basis of morphological and pharmacological observations that the heartbeat of the branchiopods *Daphnia magna* and *Daphnia pulex* is myogenic (Baylor, 1942; Bekker and Krijgsman, 1951, reviewed by Krijgsman, 1952), although electrophysiological evidence has not been reported. Recently, Yamagishi *et al.* (1993) have shown by recording intracellular

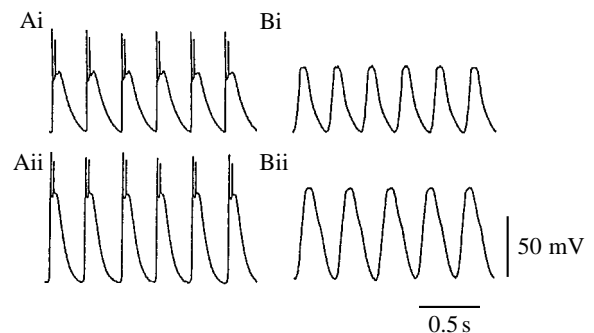
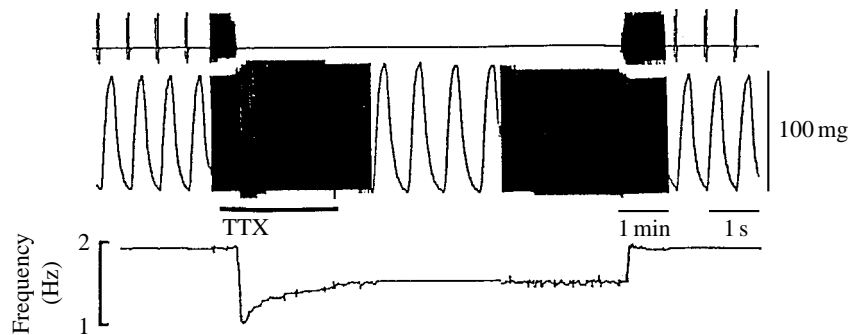


Fig. 15. Effects of current injection on muscle activity of adult hearts in normal and TTX-containing salines. Intracellular activity recorded from the heart muscle in normal (A) and TTX-containing ($10^{-6} \text{ mol l}^{-1}$) saline (B). Records under unstimulated (upper traces) and hyperpolarized (lower traces) conditions are shown. The muscle membrane was hyperpolarized by current injection (40 nA). From an immature adult 12 mm in body length.

Fig. 16. Effects of TTX on adult heartbeats. The impulse activity recorded from the cardiac ganglionic trunk (top trace), the mechanical record of the heartbeat (middle trace) and the frequency of the heartbeat (bottom trace) are shown. $10^{-6} \text{ mol l}^{-1}$ TTX was applied during the period indicated by the black bar. From an adult 32 mm in body length.



activity of the heart muscle that the heartbeat of the branchiopod *Triops longicaudatus* is myogenic.

Characteristics of the heart as a single muscle oscillator

In late embryos and early juveniles, muscle fragments isolated from all regions of the heart exhibited spontaneous bursting, indicating that there is no localized pacemaker region of the heart (Fig. 4). The muscle cells were coupled electrically and discharged almost simultaneously (Fig. 5A). These results suggest that the heart muscle cells individually possess oscillatory properties and that electrical pathways exist to coordinate their synchronous discharge. This was confirmed by cutting the cardiac ganglionic trunk. In the neurogenic heart of adult *Ligia oceanica* and *Ligia exotica*, a transverse cut of the cardiac ganglionic trunk at a central region of the heart resulted in the generation of independent burst activity in the two separated ganglia (Yamagishi and Ebara, 1985) and asynchronous beating between the anterior and posterior halves of the heart (Alexandrowicz, 1931). In contrast, in the heart of embryos and early juveniles, synchronous beating was recorded between the anterior and posterior halves of the heart after cutting the cardiac ganglionic trunk (Fig. 5B). These results indicate that the heart of late embryos and early juveniles of *Ligia exotica* has a diffuse myogenic nature and the characteristics of a functional syncytium. These features of the heart are very similar to those of molluscan myogenic hearts (reviewed by Hill and Welsh, 1966; Irisawa, 1978; Jones, 1983), especially the oyster ventricle (Irisawa *et al.* 1973; Ebara and Kuwasawa, 1975).

The heart of late embryos and early juveniles of *Ligia exotica* is a unitary muscle, as was confirmed by current injection into the heart muscle. Regardless of the injection site, the rhythm of the muscle activity was reset by a single brief depolarizing current and entrained to a higher frequency by repeated stimuli (Fig. 6). The frequency of the muscle activity changed depending on the membrane potential level at which the current was injected (Fig. 7). These responses to the applied stimuli are characteristic of endogenous oscillators in general (Pavlidis, 1973) and indicate that the heart of late embryos and early juveniles of *Ligia exotica* can be regarded as an endogenous muscle oscillator.

Cardiac ganglionic innervation in early juvenile hearts

In the myogenic heart of newly hatched juveniles, the heart

muscle produced EJP-like small depolarizing potentials following stimulation of the cardiac ganglionic trunk (Fig. 8A). The rhythm of the myogenic activity in newly hatched juvenile hearts could be reset by a single stimulus applied to the cardiac ganglion (Fig. 8C) and entrained to a higher frequency by repeated stimuli (Fig. 9). In newly hatched juveniles, many axons were identified in the cardiac ganglionic trunk (Fig. 2). In the heart of early juveniles, a pair of extrinsic cardioregulatory nerves, each containing one inhibitory and two acceleratory axons, innervates the heart muscle and can regulate the myogenic heartbeat (Yamagishi and Hirose, 1992). These cardioregulatory axons enter the heart and run through the cardiac ganglionic trunk (Yamagishi *et al.* 1989). Therefore, stimulation of the cardiac ganglionic trunk might produce compound junctional potentials on the heart muscle. However, stimulation of only the extrinsic cardioregulatory nerves at a site outside the heart failed to reset or entrain the muscle activity to a higher frequency (Yamagishi and Hirose, 1992). These results suggest that, in the heart of early juveniles, the cardiac ganglion cells have already innervated the heart muscle and have the ability to reset and entrain its myogenic activity.

Development of cardiac ganglion activity

In juveniles approximately 1 week after hatching, spontaneous EJP-like potentials appeared irregularly in the heart muscle (Fig. 10). These responses were not changed in frequency by hyperpolarizing the muscle membrane potential, in contrast to the myogenic activity (Fig. 11). Moreover, the EJP-like responses were eliminated by application of TTX (Fig. 12), which suppresses impulse generation in the cardiac ganglion (Fig. 14). These results suggest that the EJP-like responses are neurally evoked EJPs and represent the beginning of spontaneous activity of the cardiac ganglion. However, the EJPs appeared locally in the heart and no impulse activity corresponding to the EJPs was recorded from the cardiac ganglionic trunk (results not shown). This may indicate that coordinated activity is not established among the cardiac ganglion cells at this stage.

In juveniles more than 10 days after hatching, periodic bursts of impulses appeared in the cardiac ganglionic trunk (Fig. 13A). In adult hearts, six cardiac ganglion cells discharge synchronously *via* electrical connections and send periodic bursts of impulses peripherally (Yamagishi and Ebara, 1985).

The cardiac ganglion cells start spontaneous coordinated bursting during juvenile development.

Pacemaker mechanisms in late juvenile and adult hearts

Entrainment of spontaneous muscle activity by synaptic inputs from the ganglionic motor neurones has been reported in the crustacean stomatogastric system (Meyrand and Moulins, 1986; Tazaki and Miyazaki, 1991). Myogenic activity in the heart of early juveniles could also be entrained by EJPs evoked by stimulation of the cardiac ganglion (Fig. 9). When the cardiac ganglion begins spontaneous periodic bursting during juvenile development, muscle activity occurs in response to ganglionic burst discharges (Fig. 13A). The heart muscle produces periodic bursts of EJPs corresponding to the ganglionic burst discharges (Fig. 13B). These results strongly suggest that the cardiac ganglion entrains the myogenic activity to a higher frequency *via* EJPs.

This was confirmed in adult hearts by the results of TTX application. TTX suppressed impulse generation in the cardiac ganglion (Fig. 14), as reported in other crustaceans (Anderson and Cooke, 1971; Tazaki, 1971). In TTX-containing saline, the heart muscle of adults continued to exhibit endogenous oscillatory activity (Figs 14, 15). Therefore, the heartbeat of adults was changed reversibly from neurogenic to myogenic by the application of TTX, with the neurogenic heartbeat being higher in frequency than the myogenic one (Fig. 16). Although the frequency of the heartbeat decreases during growth, the heartbeat of the same preparation was generally higher in frequency in normal saline than in TTX-containing saline (Yamagishi and Mori, 1993).

In the heart of all crustaceans investigated so far, the heart muscle has no myogenicity and the cardiac ganglion acts as a dominant pacemaker (reviewed by Prosser, 1973). In the leech *Hirudo medicinalis*, Maranto and Calabrese (1984) have shown that the heart excitor motor neurones located in the central nervous system reset and entrain the heart myogenic rhythm to a higher frequency *via* EJPs. In the heart of adult *Ligia exotica*, two pacemaker sites, the cardiac ganglion and the heart muscle itself, are present, and the cardiac ganglion acts as a primary pacemaker to entrain the muscle activity to a higher frequency *via* EJPs. This type of neurogenic heartbeat is different from that known in any other crustacean, but is similar to that in the leech.

Transfer of the heart pacemaker during development

The results of the present study suggest that the pacemaker function of the heart of *Ligia exotica* is transferred from the heart muscle to the cardiac ganglion during juvenile development. As a result, the initiation of the heartbeat changed from myogenic to neurogenic but of a type different from that reported for other crustaceans and *Limulus polyphemus*.

Recently, we have shown that the heartbeat of adults of the primitive crustacean *Triops longicaudatus* (Branchiopoda) is myogenic (Yamagishi *et al.* 1993). This suggests that there is some diversity in the heartbeat pacemaker mechanism in

Crustacea. The present findings show that this diversity is present not only at the phylogenetic but also at the ontogenetic level.

We thank Dr K. Oami for helpful discussions and Dr Darryl Macer for his reading of this manuscript. This work was supported in part by a Grant-in-Aid (no. 08640857) from the Ministry of Education, Science, Sports and Culture of Japan to H.Y. Contribution no. 608 from the Shimoda Marine Research Center, University of Tsukuba.

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