

THE PACEMAKER ACTIVITY GENERATING THE INTRINSIC MYOGENIC CONTRACTION OF THE DORSAL VESSEL OF *TENEBRIO MOLITOR* (COLEOPTERA)

T. MARKOU AND G. THEOPHILIDIS*

Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University, Thessaloniki 54006, Greek Macedonia, Hellas (Greece)

*Author for correspondence (e-mail: theophil@bio.auth.gr)

Accepted 2 September; published on WWW 24 October 2000

Summary

Combined intracellular and extracellular recordings from various parts of the isolated dorsal vessel of *Tenebrio molitor* revealed some of the following electrophysiological properties of the heart and the aorta. (i) The wave of depolarization causing forward pulsation of the dorsal vessel was always transmitted from posterior to anterior, with a conduction velocity of 0.014 m s^{-1} in the heart and 0.001 m s^{-1} in the aorta when the heart rate was $60 \text{ beats min}^{-1}$. (ii) There was no pacemaker activity in the aorta. (iii) The duration of the compound action potential in the aortic muscle depended on the duration of the pacemaker action potential generated in the heart. (iv) Isolated parts of the heart continued to contract rhythmically for hours, indicating powerful pacemaker activity in individual cardiac segments. (v) There was a

direct relationship between action potential duration and the length of the preceding diastolic interval. (vi) The rhythmic wave of depolarization was dependent on the influx of Ca^{2+} . (vii) The recovery of the electrical properties of myocardial cells that had been disrupted by sectioning was rapid. (viii) In hearts sectioned into two halves, the rhythmic pacemaker action potentials recorded simultaneously from the two isolated halves eventually drifted out of phase, but they had the same intrinsic frequency. In the light of these data, we discuss two alternative models for the generation of spontaneous rhythmic pumping movements of the heart and aorta.

Key words: pacemaker, insect, heart, aorta, myogenic rhythm, *Tenebrio molitor*, mealworm beetle.

Introduction

The circulation of haemolymph in insects is maintained mainly by a muscular pump, called the dorsal vessel, which is tubular in shape and located dorsally. It consists of the heart, located in the abdomen, and the aorta, which extends through the thorax. The heart collects haemolymph from the abdominal cavity and discharges it into the head through the aorta (Chapman, 1975). The walls of the heart and the aorta are contractile and are composed of a single layer of striated muscles, arranged in an interlacing network of fibres that run both longitudinally and radially (McCann, 1963, 1965; Miller, 1985). The control of rhythmic contractions in the insect heart is a complicated process. In some insects, such as cockroaches, the heart receives innervation from three sources; however, when the heart is denervated, the contractions are still perfectly coordinated. Removal of the lateral cardiac nerve cords has only a small effect on cardio-regulation (Miller and Usherwood, 1971) and is followed by the resumption of rhythmic contractions (Miller and Metcalf, 1968). Miller and Usherwood (1971) suggested that 'a heart devoid of its lateral cardiac nerve cords still contracts in a highly co-ordinated manner. This surprising observation makes the role of the cardiac nervous system somewhat difficult to understand'. It is even more difficult to understand how contractions of different

cardiac segments are coordinated in those insect hearts that lack innervation completely, such as those of the moths *Manduca sexta* (Tublitz, 1988), *Hyalophora cecropia* (Sanger and McCann, 1968) and the mosquito *Anopheles quadrimaculatus* (Jones, 1954). In such cases, the heart beat must be myogenic.

Myogenic hearts can contract rhythmically in the absence of any nervous input. Probably the most convincing demonstration of myogenicity comes from work on the hearts of *Periplaneta americana* and *Drosophila melanogaster*, in which perfusion with tetrodotoxin eliminates all nervous activity in the lateral cardiac nerve cords and yet normal contractions continue (Smith, 1969; Gu and Sing, 1995).

The electrophysiological properties of myogenic hearts in insects have been described by a number of workers (McCann, 1962, 1963; Miller, 1969, 1974, 1985; Bruen and Ballard, 1970; Tadmowski and McCann, 1980; Richter and Hertel, 1997). In *Drosophila melanogaster*, several mutations that affect ion channel function directly or indirectly have been screened for effects on the electrophysiological properties of the heart. For example, mutations such as 'no action potential temperature sensitive' (nap^{ts}) (Wu et al., 1978; Wu and Ganetzky, 1980) induce arrhythmia in the heartbeat of *D.*

melanogaster larvae at temperatures above 20 °C (Dowse et al., 1995). However, despite these studies, the arrangement and location of the pacemaker or pacemakers generating myogenic activity in insect hearts remain unclear.

In an attempt to add to the existing information on the complicated system of pacemakers that control myogenic contractions of the dorsal vessel in insects, we have investigated the electrophysiological properties of the heart and the aorta of a coleopteran, the mealworm beetle *Tenebrio molitor*.

Materials and methods

The experiments were performed on adult *Tenebrio molitor* (L.), of either sex, reared in laboratory colonies and fed on bran, potatoes and fruit under controlled conditions of temperature (30±1 °C) and photoperiod (16 h:8 h light:dark). Under these conditions, the last instar lasted 14±0.6 days (mean ± s.d., *N*=30). The pupal stage, from the formation of the pupa until emergence of the adult, lasted 6±0.3 days (*N*=35). We conducted experiments on final-instar larvae that were 3–5 days old, pupae that were 3–4 days old and adults that were at least 10 days old.

For anatomical and physiological studies, *T. molitor* of all three stages were dissected as follows. The wings and legs (for the adult) were removed, and the insect was mounted, using fine dissecting pins, dorsal side down in a small recording chamber. An incision was made along the midline of the abdomen and thorax, and the two sides of the body wall were pinned down laterally. The gut, glands and abdominal muscles were removed to expose the dorsal vessel (heart and aorta) and the alary muscles attached on the dorsal region of the abdomen. The thorax and abdomen were continuously superfused with oxygenated physiological saline (in mmol l⁻¹: NaCl, 80; KCl, 5; CaCl₂·2H₂O, 0.2–2; saccharose, 90; glucose, 10; and Hepes, 20; pH 6.7) at a flow rate of 1–1.5 ml min⁻¹. Under these recording conditions, the heart and aorta continued to contract spontaneously for more than 15 h.

Extracellular recordings from the aorta were obtained using a suction electrode made from a glass micropipette. The tip of the micropipette was broken so that the inside diameter was slightly smaller than the outside diameter of the aorta (34–64 µm), and part of the aorta was sucked up. The spontaneously generated aortic compound action potentials (CAPs) were amplified using a low-noise, high-input-impedance differential alternating current preamplifier (Neurolog 104, NL115). Suction electrodes were also used to record extracellular potentials from the myocardium of the insect heart (Yeager, 1939; McCann, 1969; Ballard and Hall, 1969). Intracellular recordings from the heart and the aorta were obtained using floating glass microelectrodes, filled with 2 mol l⁻¹ potassium acetate (resistance 40–60 MΩ). Floating microelectrodes are ordinary microelectrodes that are not fixed onto the probe of the amplifier (Neurolog NL102), but are suspended from the fine silver wire of the probe. They penetrate the muscle fibre and are able to follow its movements

without breaking. Similar electrodes have been used to record spontaneous motor activity from respiratory muscles or the oviduct in Orthoptera (Consoulas and Theophilidis, 1992; Kalogianni and Theophilidis, 1994).

Muscle tension in the aorta was monitored using a force displacement transducer (Grass FT83C). A microelectrode attached to the arm of the transducer was hooked onto the posterior end of the aorta (see inset of Fig. 2A) using a micromanipulator. Using the micromanipulator, the aorta was stretched until its contractions produced measurable forces at the transducer.

All recordings were fed into a digitizing oscilloscope with an interface circuitry that allowed the recordings to be transferred to a personal computer. Experiments were automated using customized software that sampled and stored the data and computed duration and latency measurements. The duration of the depolarization recorded intracellularly from two myocardial cells was defined as the time interval between the points marked as 50% of the rising phase and 50% of the falling phase. The latency between two intracellular action potentials recorded from two different parts of the dorsal vessel was defined as the time interval between the points marked as 50% of the rising phase of successive depolarizing phases. Results are presented as means ± s.d.

For the anatomical studies, the heart, aorta and alary muscles were stained with Toluidine Blue, fixed in Carnoy fixative and dehydrated through a series of alcohols. They were observed under the light microscope, and drawings were made using a *camera lucida*.

Results

Morphology of the dorsal vessel

In adult *T. molitor*, the heart is 0.08–0.13 mm wide, 4.25±0.30 mm (mean ± s.d., *N*=10) long (Fig. 1A) and has no chambers or valves. Closely associated with the heart are six pairs of alary muscles (Fig. 1A) and six pairs of ostia or openings. The alary muscles fan out from restricted origins on the abdominal segments, the muscle fibres of each side meeting in a broad zone at the heart. In this study, the alary muscles were used to divide the heart into six different regions termed cardiac segments (see Fig. 1).

In the last larval stage, the heart is 0.17–0.29 mm wide and 9.06±1.17 mm long (mean ± s.d., *N*=9). The muscle fibres of the alary muscles appear more delicate and they are smaller in number. In the larval stage, there are 18±6 (mean ± s.d., *N*=9) muscle fibres for each pair of alary muscles, whereas in the adult the number increases to 25±8 (mean ± s.d., *N*=10). A typical cardiac segment is shown in Fig. 1B. The larval heart also lacks chambers or valves. Fig. 1A,B shows that there are clear differences in the diameter of the heart and the organization of the alary muscles between the larval and the adult stages of *T. molitor*. This is an indication that the dorsal vessel changes drastically during the 7-day pupal stage. Some anatomical features of the heart in the pupal stage (3–4 days old) are shown in Fig. 1C. In this case, it is interesting that the

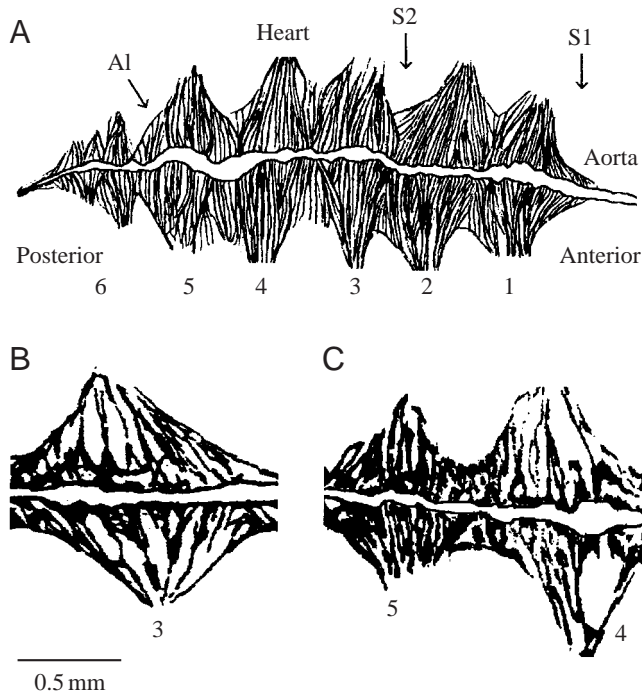


Fig. 1. *Camera lucida* drawings of the heart and alary muscles (Al) of *Tenebrio molitor*. (A) Adult stage; (B) last larval stage; (C) pupal stage (3–5 days). S1, S2 are the points where the heart was sectioned in the physiological experiments (see text). The numbers indicate the pairs of alary muscles and different cardiac segments.

alary muscles in the posterior cardiac segments (see fifth segment in Fig. 1C) have a very similar anatomy to those of the adult stage, whereas those in the anterior segments (see fourth segment in Fig. 1C) have an anatomy that more closely matches that of the larval stage. Thus, these changes in the morphology appear to start in the posterior segments and finish in the anterior segments of the heart during metamorphosis. The aorta, a small part of which is shown in Fig. 1A, is a muscular tube that runs along the thorax to the head. The aorta is 0.92 ± 0.03 mm (mean \pm S.D., $N=9$) long in the adults and 3.12 ± 0.58 mm (mean \pm S.D., $N=7$) in the larvae. In this study, we do not give details of the innervation pattern of the heart or aorta, since our focus was to investigate in detail the intrinsic myogenic activity only. To eliminate any possible effects of neural inputs on the intrinsic myocardial activity, we removed the whole central nervous system in all the electrophysiological experiments performed in this study (see also Miller, 1985).

The physiological properties of the aorta

A simple way to locate the presence of pacemaker activity in the heart was to section the dorsal vessel transversely at different levels and observe the contractions of the isolated parts (see also Miller, 1974). For example, after a transverse section anterior to the first cardiac segment, at the point where the heart and the aorta are connected (S1 in Fig. 1A), contractions of the aorta were completely eliminated. In three out of ten preparations, there were slight movements of the

isolated aorta, but they were negligible compared with the original powerful contractions of the aorta in the intact preparation. These experiments show that there are no pacemaker cells present within the aorta that are powerful enough to generate and maintain the rhythmic contractions seen in the intact preparation. Thus, the contractions of the aorta are driven by electrical activity generated somewhere in the main part of the heart. After sectioning at S1, the rest of the heart maintained the normal cycle of contractions, which started from the sixth and ended at the first cardiac segment.

In a different preparation, after sectioning the heart between the second and third segments (see S2, in Fig. 1A), the two remaining parts, segments 1–2 and segments 4–6, continued to contract rhythmically. Furthermore, in the isolated posterior part of the heart, the sequence of activation remained the same. The sixth segment always contracted first, followed by contraction of each anterior segment in turn. When the tubular heart was cut into five parts, the individual pieces continued to contract rhythmically for at least 2 h. Similar results were obtained in 10 other experiments, indicating the presence of pacemaker activity within each of the isolated cardiac segments, but not in the aorta.

For a detailed study of the electrophysiological properties of the aorta, two electrodes were used. The first, a microelectrode (electrode II, in the inset of Fig. 2), was used to record intracellularly the muscle action potentials in individual heart muscle fibres in the region where the heart and the aorta join. The second, a suction electrode, was placed over the free end of the aorta (see electrode I in the inset of Fig. 2) to record extracellularly the electrical activity of the group of aortic muscle fibres. Simultaneous recordings, using the two electrodes, are shown in Fig. 2A. Pacemaker action potentials were recorded intracellularly from a single myocardial cell located at the heart/aorta connection (Fig. 2A, upper trace). Each pacemaker potential consisted of a gradual initial depolarization leading to a rapid and sustained depolarization that was eventually terminated by a rapid repolarization. These pacemaker potentials preceded and were phase-locked to the electrical activity recorded extracellularly from the other end of the aorta (Fig. 2A, lower trace), with a constant delay of 0.15–0.19 s. The wall of the aorta is composed of a single layer of striated muscle fibres arranged in an interlacing network that runs longitudinally and radially (McCann, 1963; Miller, 1985; Rugendorff et al., 1994). The electrical signal recorded using the suction electrode (Fig. 2A, lower trace) represents the wave of depolarizations generated in a number of aortic muscle fibres. In this study, because the waves of depolarization of the aortic muscle fibres were generated at almost the same time, we refer to the recorded electrical deflection as the aortic compound action potential (aortic CAP).

Because all electrical activity in the aorta was eliminated as soon as the aorta was separated from the heart, the recordings in Fig. 2A show that the pacemaker action potential of the heart (Fig. 2A, top trace) was responsible for the compound action potential (CAP) recorded from the aorta (Fig. 2A, lower

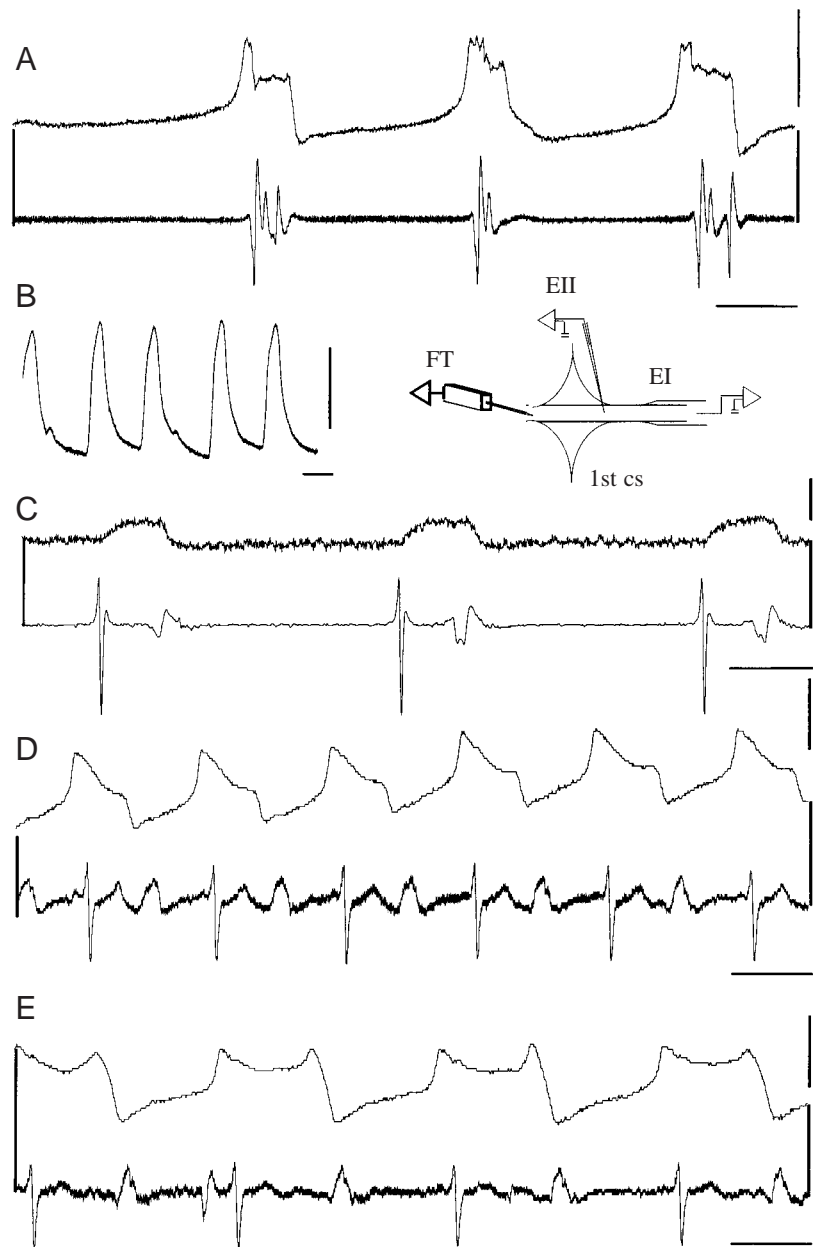


Fig. 2. Recordings from various parts of the isolated dorsal vessel of *Tenebrio molitor*. (A) Top trace: intracellular recordings from the first cardiac segment (1st cs, electrode II in inset; EII); Lower trace: extracellular recording from the aorta using a suction electrode (see electrode I in the inset; EI). Scale bars: vertical, 10 mV; horizontal, 1 s. (B) Intracellular recording from ordinary heart muscle fibres that lack pacemaker properties. Scale bars: vertical, 50 mV; horizontal, 1 s. (C) Top trace: muscle tension recorded from the first cardiac segment of the heart. Lower trace: extracellular recording from the aortic muscle fibres. The suction electrode was used to record the electrical activity of the aorta and to restrain the heart to record the muscle tension (see inset). FT, force transducer. Scale bars: vertical, 1 mg; horizontal, 1 s. (D,E) As for A, but after the preparation had been left to settle for 1 h. Note the difference in the heart rate between D and E. Scale bars: vertical, 10 mV; horizontal, 1 s.

trace). Sometimes, usually at the beginning of the experiment (see also Bruen and Ballard, 1970), there were distinct differences in the duration of the pacemaker action potentials (see Fig. 2A top trace). In the experiment shown in Fig. 2A, whenever the duration of the pacemaker action potential increased (top trace), the duration of the aortic CAP also increased (lower trace). Analysis of this recording showed that, when the pacemaker action potential had a minimum duration of 0.46 s, the duration of the aortic CAP was 0.54 s, whereas the longest pacemaker action potential of 0.98 s corresponded to an aortic CAP of 1.02 s. The direct relationship between the duration of the pacemaker action potential and the duration of the aortic CAP is supported by linear regression analysis (goodness of fit $r^2=0.786$, $P=0.020$, $N=16$).

Normal muscle action potentials (with no pacemaker

activity) were recorded from almost every muscle fibre of the aorta (Fig. 2B). There was always a 1:1 relationship between the aortic CAP and a contraction of the aorta (Fig. 2C).

In other experiments, when the preparation was left to settle for over an hour, the relationship between the pacemaker action potential recorded from the first cardiac segment and the aortic CAP (see Fig. 2D,E) was more constant. In some experiments, we increased the frequency of the cardiac rhythm by injecting positive current through the microelectrode for a short period (1–2 s), and observed an interesting phenomenon. When the heart rate increased from 18 to 60 beats min^{-1} , the duration of the aortic depolarization decreased approximately threefold, from 1.70 ± 1.20 s (mean \pm s.d., $N=10$) to 0.51 ± 0.03 s (mean \pm s.d., $N=18$). In contrast, the conduction velocity of the aortic CAP increased from 0.001 to 0.002 m s^{-1} . These data

come from a single experiment; the results from three other experiments were very similar.

The location of the aortic pacemaker

When we sectioned the heart anterior to the sixth or third cardiac segments or half-way through the first cardiac segment (S1, S2 and S3 in the inset of Fig. 3), there was no effect on

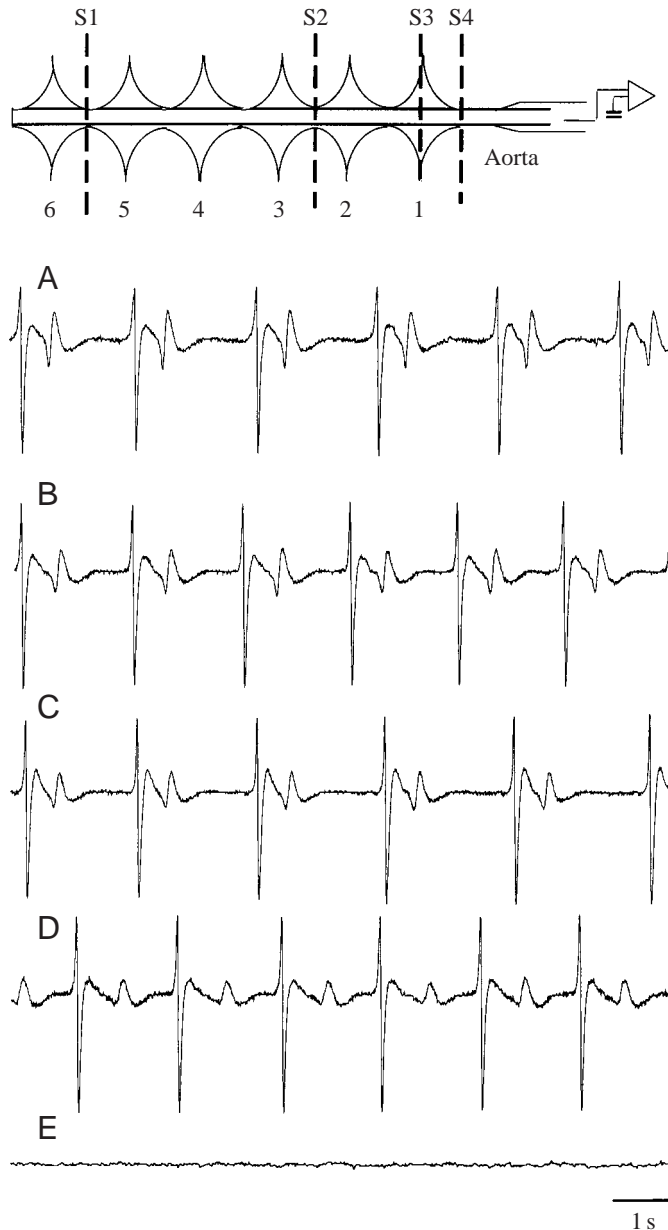


Fig. 3. Extracellular recordings from the aorta of the dorsal vessel of *Tenebrio molitor* made using a suction electrode (see inset). During the course of the experiment, the heart was progressively sectioned at the levels shown in the inset (S1–S4). The numbers 1–6 indicate the pairs of alary muscles and the different cardiac segments. (A) Intact heart. (B) Heart sectioned at S1 (see inset). (C) Heart sectioned further at S2. (D) Heart sectioned further still at S3. (E) Aorta separated from the heart after sectioning at S4. All activity in the aorta has ceased.

the intrinsic rhythm of the aorta. Detailed study of the recordings revealed a small effect on either the amplitude or the shape of the recorded aortic CAP, which we discuss below (compare Fig. 3A, the control, with Fig. 3B–D). However, when the region that joins the first cardiac segment to the aorta, the heart/aorta connection, was cut (S4 in the inset of Fig. 3), all activity in the aorta ceased instantly and never recovered (Fig. 3E).

This experiment showed that the firing rate of the aorta was not affected by the removal of most of the heart as long as it was still attached to the first cardiac segment. However, detailed examination of the recordings showed that, each time the heart was sectioned, there were small changes in either the amplitude or the shape of the aortic CAP but never in its frequency. These changes in shape probably reflected the number of excited aortic muscle fibres that contributed to the aortic CAP. For example, in one experiment, immediately after the removal of the fifth and sixth cardiac segments, the peak amplitude of the depolarizing phase of the aortic CAP was reduced to approximately 67% of control levels (compare Fig. 4Ai, before the section, with Fig. 4Aii, immediately after the section). There were no further changes in the electrical activity of the aorta (the aortic CAP) after the removal of the third and fourth cardiac segments (Fig. 4Aiii). In other experiments, changes in the aortic CAP caused by injury to the heart were less obvious (see Fig. 4B).

These small changes in the aortic CAP, which reflect changes in the pacemaker input to the aorta, were also demonstrated by recording the aortic CAP continuously during the sectioning experiments. When the fifth and sixth cardiac segments were removed, the shape of the action potential changed immediately (compare Fig. 4Cii, before sectioning, with Fig. 4Ciii, after sectioning), as expected from the previous experiment. Recordings such as those shown in Fig. 4Ciii were obtained for more than 20 min. When the second and third segments were cut, there were some drastic changes in the aortic CAP (compare Fig. 4Ciii with Civ). The shape of the aortic CAP recovered gradually over the next 2–3 min (Fig. 4Cv,vi). These types of change were observed in three out of four preparations. The effects on the shape of the aortic CAP (Fig. 4Civ,v) indicated that sectioning had a drastic action on the repolarization phase of the depolarizing wave (see also Fig. 5), which lasted only a few minutes. The repolarizing phase of the aortic CAP recovered rapidly, probably because of the fast recovery of the injured pacemaker cell.

The effects of Ca^{2+} on the aortic pacemaker

Intracellular recordings from a pacemaker cell at the heart/aorta connection in control saline are shown in Fig. 5A. The mean duration of the pacemaker action potential was 451 ± 0.17 ms (mean \pm s.d., $N=20$, $66 \text{ beats min}^{-1}$) and the mean amplitude of the depolarizing and repolarizing phases was 11.39 mV ($N=45$). After application of 0.02 mmol l^{-1} EGTA, which removed the influence of Ca^{2+} , there was a gradual increase in the mean duration of the pacemaker action potential from 451 ± 0.17 ms (Fig. 5A) to 1.08 ± 0.32 s (means

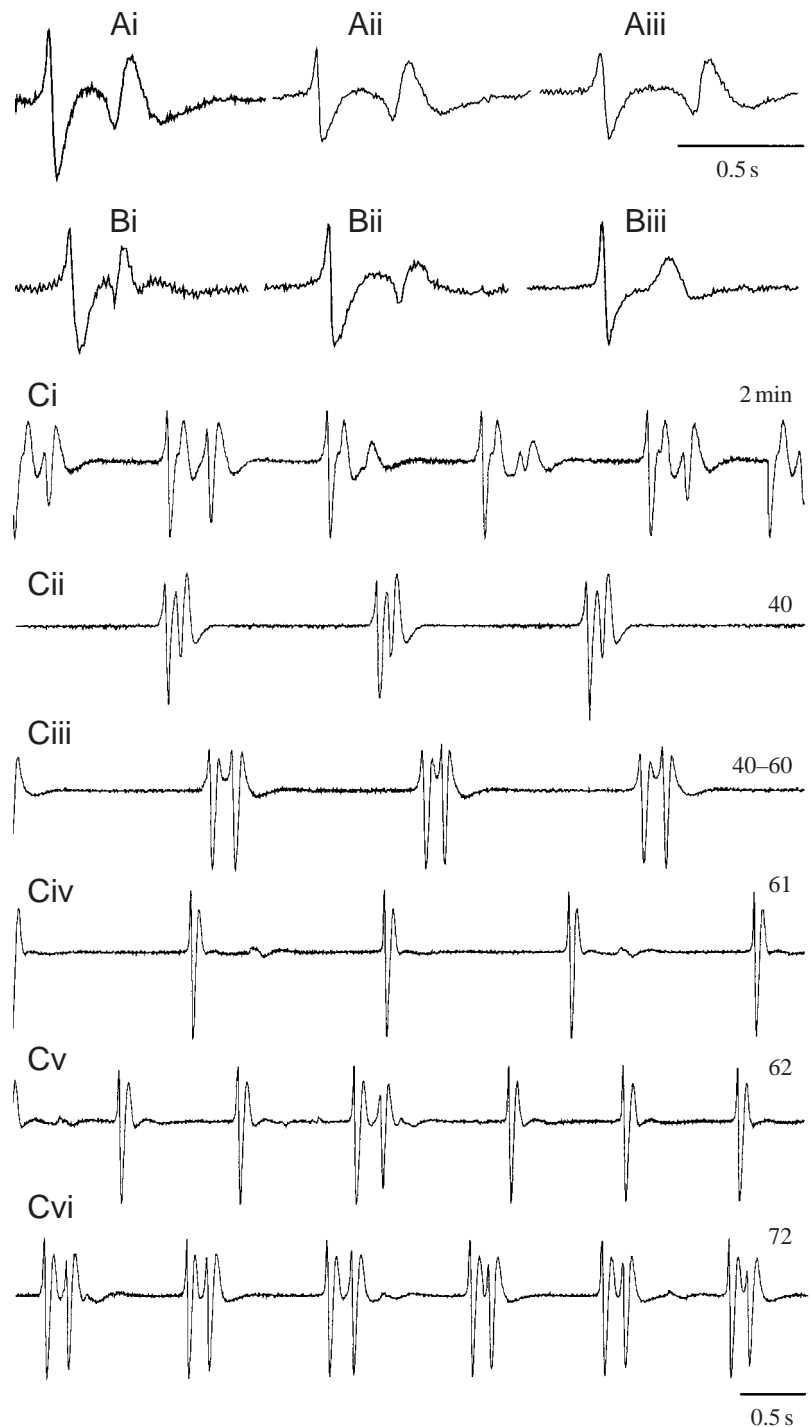


Fig. 4. (A,B) Recordings of the aortic compound action potential from an isolated dorsal vessel of *Tenebrio molitor*. The expanded traces in A and B are from two different experiments. (Ai,Bi) Control conditions; (Aii,Bii) after the first section, in which the fifth and sixth cardiac segments were removed; (Aiii,Biii) after the second section of the heart, in which the third and fourth cardiac segments were removed (see text for details). (Ci,Cii) Recordings of the aortic compound action potential of an isolated intact dorsal vessel; (Ci) 2 min after dissection; (Cii) 40 min after dissection; (Ciii) after the last part of the heart, the fifth and sixth cardiac segments, had been removed. Recordings such as these were obtained for more than 20 min (40–60 min). (Civ) Recording after the remaining second and third cardiac segments had been removed. (Cv) Within 1 min (see 62 min) after this section, aortic activity is recovering. (Cvi) The aortic activity had recovered completely in approximately 10 min (see 72 min), although more than 90% of the heart had been removed.

\pm s.d., $N=20$) (Fig. 5B) in the first 2 min and from 1080 ± 320 to 3150 ± 1480 ms (means \pm s.d., $N=20$) in the next 2 min (Fig. 5C). Eventually, 3–4 min after the application of EGTA, the amplitude of the depolarizing phase was close to that recorded in control saline (in control saline, 11.39 ± 1.56 mV, $N=45$; in EGTA, 11.09 ± 0.98 mV; means \pm s.d., $N=45$), but the cell failed to repolarize (see Fig. 5D,E). Repolarization occurred immediately when the preparation was washed in control saline containing $0.2 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ (see arrow in Fig. 5E). The pacemaker action potential recovered to control

values (see Fig. 5A) in less than 5 min. A similar response to Ca^{2+} -free saline was recorded from pacemaker myocardial cells in three other experiments. The small size of the aorta ($0.034\text{--}0.064$ mm in diameter) and its rhythmic contractions make penetration of individual muscle fibres very difficult in this type of experiment, even with floating electrodes.

These experiment clearly indicate that the elimination of the repolarizing phase of the pacemaker action potential observed when the heart was sectioned (see above) matched the response of myocardial cells to Ca^{2+} -free saline.

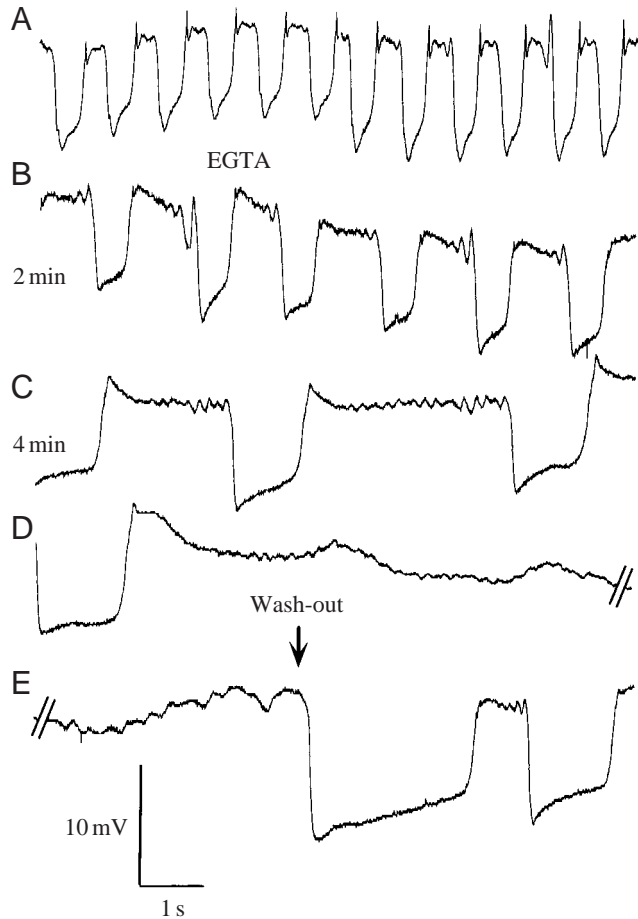


Fig. 5. Intracellular recordings from a single pacemaker cell in the heart/aorta connection of *Tenebrio molitor*. (A) Preparation bathed in control saline (containing $0.2 \text{ mmol l}^{-1} \text{ Ca}^{2+}$). (B–D) Effects of applying 0.02 mmol l^{-1} EGTA for 6 min. (E) Wash-out of EGTA with control saline.

Pacemaker activity in the heart

For a detailed study of the electrophysiological properties of the heart muscle, action potentials were recorded intracellularly from several parts of the heart (electrodes I, II and III in the inset of Fig. 6). Two types of action potential were recorded. The first type, which we have described previously and termed the pacemaker action potential, was recorded in almost every cardiac segment (see, for example, Fig. 6B upper trace and Fig. 6C lower trace). The second type, which we have termed the normal cardiac muscle action potential, lacked the initial gradual depolarization and consisted of a rapid and sustained depolarization followed by a rapid repolarization (see, for example Fig. 6A,C, upper traces). Intracellular recordings from myocardial cells showed clearly that the heart has both types of potential in nearly all cardiac segments.

The combined intracellular recordings from the heart and extracellular recordings from the aorta showed that there was perfect correspondence between the action potential generated in the muscle fibres of the cardiac segments and the aortic CAP

(see Fig. 6A,B). These potentials were phase-locked with a constant time delay, 163 ms for the fourth cardiac segment and 180 ms for the sixth cardiac segment. To measure the conduction velocity of the travelling depolarizing wave in the heart, simultaneous intracellular recordings from both ends of the heart were obtained (Fig. 6C). Pacemaker depolarizations, which had a mean duration of $0.69 \pm 0.08 \text{ s}$ (mean \pm s.d., $N=28$), were recorded in the sixth cardiac segment and were followed by muscle action potentials of the same duration in the first cardiac segment with a delay of $0.267 \pm 0.023 \text{ s}$ (mean \pm s.d., $N=32$). According to detailed anatomical studies (Fig. 1A), the distance between the two electrodes should have been 3.8–4 mm, so the conduction velocity along the heart is between 0.014 and 0.015 m s^{-1} .

The perfect coordination between the pacemaker action potentials generated in the muscle fibres of the last (sixth) cardiac segment and the muscle compound action potentials recorded from the aorta is also shown in Fig. 7A. Here, the time delay between the two potentials was $0.183 \pm 0.007 \text{ s}$ ($N=60$). In the same preparation, when the heart was cut between the third and fourth segments (see section S in the inset of Fig. 7), the aorta maintained its original intrinsic rhythm (Fig. 7B lower trace) with some minor changes in the amplitude of the aortic CAP, which have been discussed previously. However, there were drastic but temporary changes in the activity of the recorded cell in the sixth cardiac segment, which lay in the part isolated from the remaining dorsal vessel. The amplitude of the fast depolarization decreased from $8.96 \pm 1.22 \text{ mV}$ ($N=10$) to $6.22 \pm 2.09 \text{ mV}$ ($N=8$) (i.e. a reduction of 30%), whilst its duration increased from $0.50 \pm 0.06 \text{ s}$ to $4.70 \pm 0.68 \text{ s}$ ($N=7$) (compare upper traces of Fig. 7A,B). These changes did not last long, since the intracellular recordings obtained from the isolated part after 10–15 min showed that recovery, which was marked as a sustained decrease in the duration (see Fig. 7C), had occurred. Note that the effects of sectioning the heart on the intracellular recording of muscle fibre were very similar to the response of a myocardial cell exposed to Ca^{2+} -free saline (compare Fig. 7B with Fig. 5).

In all four experiments, the aorta and the sixth cardiac segment isolated from each other continued to contract rhythmically for at least another 2 h. After it had recovered from the sectioning, the isolated posterior half of the heart was activated at almost the same rate as the anterior half and the aorta (compare the upper and lower traces in Fig. 7C). Similar results were obtained in all four experiments.

This interesting observation was verified in another type of experiment in which the heart was also sectioned between the third and fourth segments, but this time the two fragments of the heart were placed in separate recording chambers, in separate Faraday cages, and recordings were obtained using different amplifiers. Fig. 7D shows that, although pacemaker action potentials generated by the two isolated parts of the heart appeared to coincide over short periods, they gradually drift out of phase (see dashed lines). Nevertheless, measurements of the frequency of over 100 consecutive

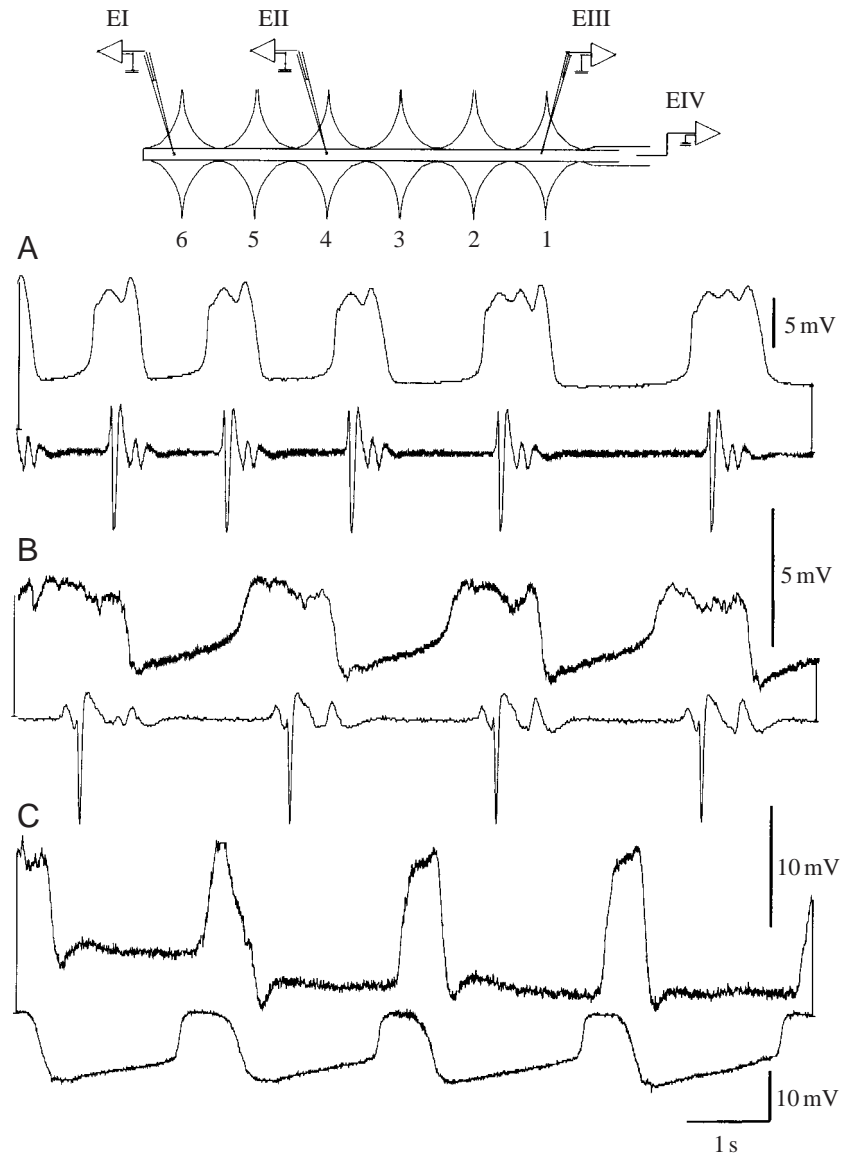


Fig. 6. Combined intracellular and extracellular recordings from the dorsal vessel of *Tenebrio molitor*. The numbers 1–6 indicate the pairs of alary muscles and different cardiac segments. (A) Top trace: intracellular recordings from the fourth cardiac segment (electrode II in the inset; EII). Lower trace: extracellular recordings from the aorta using a suction electrode (electrode IV in inset; EIV). (B) Top trace: intracellular recordings obtained from the sixth cardiac segment (electrode I in inset; EI). Lower trace: extracellular recordings from the aorta using a suction electrode (electrode IV in inset). (C) Top trace: intracellular recordings from the first cardiac segment (electrode III in inset; EIII). Lower trace: intracellular recordings from the sixth cardiac segment (electrode I in inset).

pacemaker action potentials from both isolated parts showed that the mean frequency of potentials recorded from the posterior (caudal) part and the anterior part of the heart was very similar. Statistical analysis of the data (unpaired *t*-test) showed that there was no significant difference between the mean frequency of the pacemaker action potentials in the two isolated halves of the heart ($P=0.4698$, $N=35$). A crucial condition in this experiment was the constant flow of oxygenated saline in both recording chambers.

Discussion

The insect dorsal vessel, comprising the heart and aorta, functions as an effective pump that pushes the haemolymph from the last abdominal segment to the head. This physiological function is called forward pulsation. In *T. molitor*, combined intracellular and extracellular recordings from various parts of the isolated dorsal vessel have revealed

the following electrophysiological properties of the heart and the aorta during forward pulsation.

(i) The wave of depolarization causing forward pulsation was always transmitted from the most posterior cardiac segment to the most anterior part of the dorsal vessel.

(ii) There was no pacemaker activity in the aorta. The aorta was driven by a wave depolarization, originating in the heart, where it was transmitted with a constant conduction velocity of 0.014 m s^{-1} . As soon as the depolarization reached the first cardiac segment, it stimulated the muscle fibres of the aorta electrically, generating forward pulsation of the aorta, which has a much slower conduction velocity of 0.001 m s^{-1} . Thus, the aorta consists only of ordinary muscle fibres that lack pacemaker properties.

(iii) The duration of the aortic CAP depended on the duration of the pacemaker action potential generated by the heart. There was perfect coordination between the contraction of the cardiac segments and the aorta. This shows clearly that

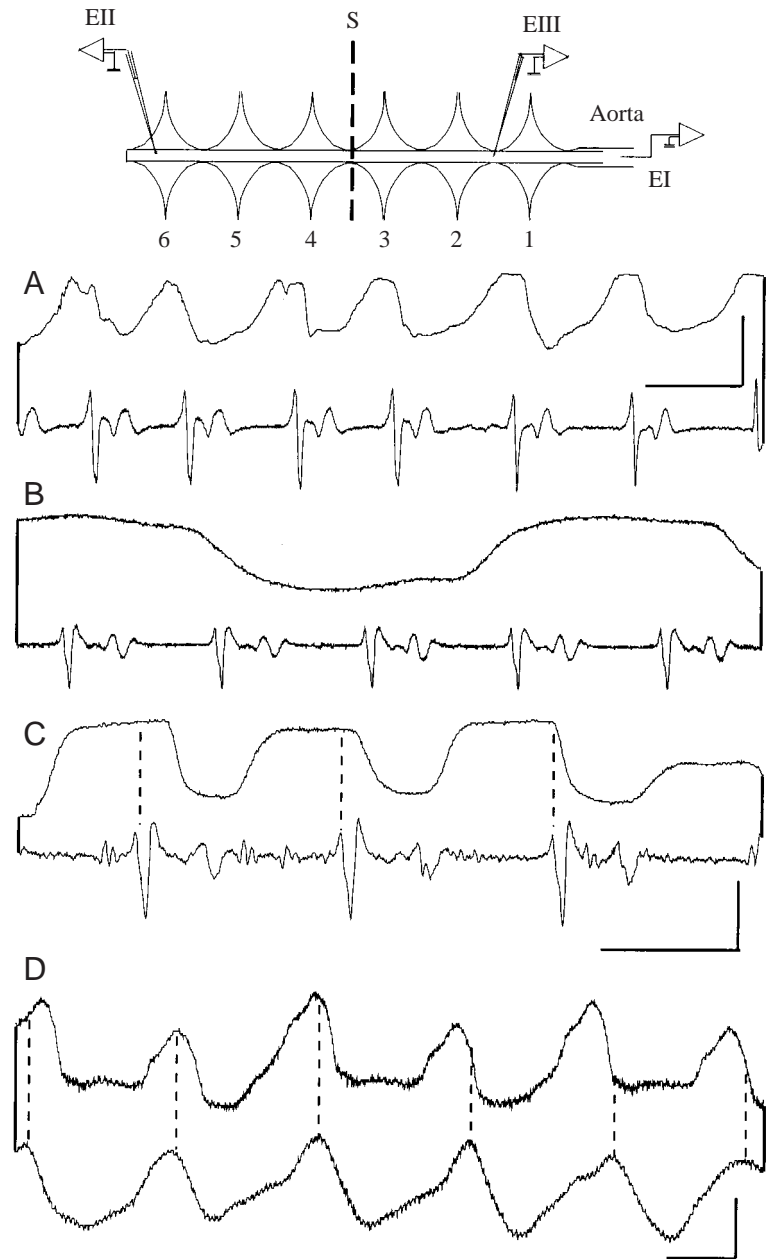


Fig. 7. Combined intracellular and extracellular recordings from various parts of the dorsal vessel of *Tenebrio molitor*; the numbers 1–6 indicate the pairs of alary muscles and the different cardiac segments. (A) Top trace: intracellular recording from the last (sixth) cardiac segment (see electrode II in inset; EII). Lower trace: extracellular recording of the aortic muscle fibres (see electrode I in inset; EI). Scale bars: vertical, 20 mV; horizontal, 0.5 s. (B) As in A, but immediately after the heart had been sectioned (see S in inset). (C) As in B, but 10–15 min after the section. Scale bars in B and C: vertical, 10 mV; horizontal, 0.5 s. (D) Intracellular activity from the two isolated halves of the heart, 50 min after the section between the fourth and third segments. Top trace: intracellular recording from the last (sixth) cardiac segment (electrode II in the inset; EII). Lower trace: intracellular recording from the second cardiac segment (electrode III in the inset; EIII). The broken lines indicate that the pacemaker action potentials drift out of phase. Scale bars: vertical, 20 mV; horizontal, 0.5 s.

there is electrical coupling (possibly a gap junction) between the heart and the aorta.

(iv) Isolated parts of the heart continued to contract rhythmically for hours, indicating the powerful pacemaker activity in individual cardiac segments. This seems to be a characteristic property of insect hearts because similar results are obtained when the heart is sectioned in at least three other insect species (Miller, 1974).

(v) The duration of the aortic CAP was shorter at higher heart rates, indicating a direct relationship between action potential duration and the length of the preceding diastolic interval. This property has also been found in other insect hearts (Bruen and Ballard, 1970) and it is a characteristic property of the mammalian heart (Katz, 1992).

(vi) The rhythmic pacemaker potentials depend on the influx

of Ca^{2+} . This is true of many other heart pacemakers in insects (McCann, 1963, 1971; Miller, 1985) and vertebrates (Sperelakis and Banks, 1993). In our experiments with Ca^{2+} -free saline, the depolarization phase of the pacemaker activity increased gradually in duration until it was finally replaced by an almost continuous depolarization of the membrane. In similar experiments on moths, pacemaker depolarization increases in duration to approximately seven times its control value (McCann, 1963). According to a recent model (Johnson et al., 1998), such a pacemaker could be constructed from a leaky channel that allows slow depolarization of the membrane, which would trigger a Ca^{2+} spike. The influx of Ca^{2+} subsequently triggers a Ca^{2+} -gated K^{+} channel to open, allowing K^{+} to exit the cell and reverse the depolarization. A refractory period (diastasis), which determines the rate of

pacemaker activity, would intervene before the leak current that initiates the next cycle.

In *T. molitor*, the permanent depolarization of the pacemaker action potential in Ca^{2+} -free saline indicated that there was insufficient Ca^{2+} influx to trigger opening of the Ca^{2+} -gated K^+ channels that reverse the depolarization. This is supported by the observation that repolarization occurs as soon as Ca^{2+} is added to the saline.

The recorded pacemaker cells had the ability to recover their functional properties quickly, within 1–3 min after the injury caused by the section. When the heart was cut into two pieces, the depolarization phase was greatly prolonged. This prolongation was very similar to that observed after the removal of Ca^{2+} from the saline. It is possible that the cut end of the pacemaker cell allowed Ca^{2+} to flow into the interior of the cell. Given the very tight control of intracellular $[\text{Ca}^{2+}]$ in intact cells, this Ca^{2+} influx might disrupt the membrane potential and the Ca^{2+} spikes involved in pacemaker action potentials. However, it seems that the pacemaker cells recovered their function, the generation of pacemaker action potentials, within 1–3 min of the injury. This may indicate that, over this period, the cells were able to close off their cut ends and pump out or sequester the cytosolic Ca^{2+} .

A final interesting point was that, after the heart had been sectioned into two halves, the pacemaker action potentials recorded simultaneously from the two isolated halves had the same frequency, although they drifted in and out of phase. There was no significant difference between the mean values of frequency of pacemaker action potentials produced by the two separated halves of the heart.

A model with a series of myocardial cells with pacemaker properties

This model is based on a series of six or more myocardial cells with pacemaker properties located along the heart, but not in the aorta, and connected *via* gap junctions (see Fig. 8A). Almost all the literature supports the theory of a large number of non-localized myocardial cells with pacemaker properties spread along the heart. For example, Miller and Usherwood (1971) have suggested that each muscle fibre normally undergoes endogenous rhythmic activity and that coordination between fibres is achieved partly through the electrical coupling between fibres. The model with a series of pacemaker cells connected *via* gap junctions shown in Fig. 8A can explain the maintained contraction of individual isolated heart fragments and the wave of depolarization transmitted along the heart that causes the forward pulsation. However, this model assumes that each individual pacemaker cell is driven by its previous pacemaker and, therefore, that they are all driven by a primary pacemaker located in the most posterior cardiac segment, which obviously determines the heart rate. The necessity of a primary pacemaker located in the posterior region of the heart has been proposed by other authors. Wigglesworth (1974) claimed that all segments of the heart may show autorhythmicity, but that it is normally best developed at the

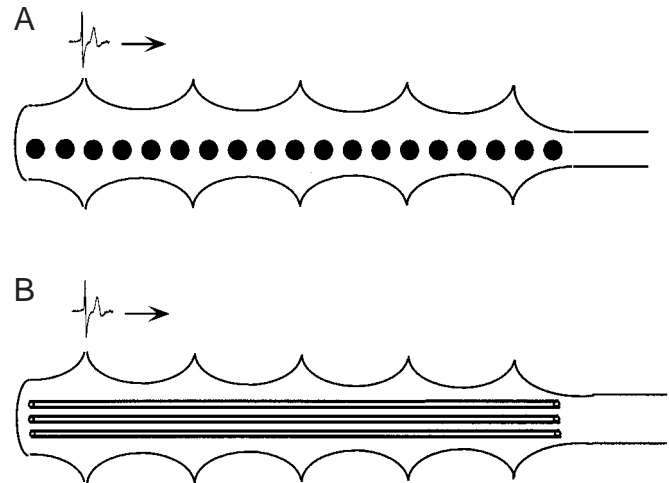


Fig. 8. Two possible mechanisms involved in the control of the spontaneous rhythmic contractions of the dorsal vessel of *Tenebrio molitor*. (A) A model with a series of individual myocardial cells with pacemaker properties. (B) A model of single longitudinal differentiated muscle fibres with pacemaker properties running along the length of the heart.

hind end. This sets the pace, and depolarizing waves pass forward from the hindmost chamber.

Unfortunately, this simple model (Fig. 8A) has to be rejected, since it does not fit with most of the electrophysiological properties of the heart found in the present study. For example, after removal of over 90% of the heart by sectioning, including the primary pacemaker as defined by this model, there were no changes either in the original frequency of the aortic CAP or in its amplitude. This model also struggles to explain how intracellularly recorded pacemaker activity from two completely isolated parts of the heart can have the same frequency. According to the model, the region of the heart posterior to the section should be driven by the primary pacemaker, whilst that anterior to the section should fire at its own intrinsic frequency. This is the case in vertebrates, in which the heart is equipped with more than one pacemaker, but the fastest pacemaker sets the pace for the whole heart (in a mammalian heart, the hierarchy of pacemaker activity is: sinoatrial node, atrioventricular node and then His bundle; Katz, 1992). Thus, the idea of many pacemakers along the heart, connected *via* electrical synapses or intercalated discs, is practically untenable. Other authors have reached the same conclusion. McCann (1965) expressed her doubts about the presence of synaptic transmission between adjacent cardiac cells, and Tadmowski and McCann (1980) claimed that autorhythmicity and electrical activation of the myocardium in the moth occur before intercalated discs with septate or other gap-type junctions are formed and in the absence of ganglionic connectives. In an attempt to explain how coordination of the insect heart is achieved, they proposed that synchronization of cardiac cell excitation and contraction in the moth is effected by a process that involves either mechanical stretching or capacitative coupling.

A model with longitudinal differentiated muscle fibres with pacemaker properties

In an attempt to find a more successful explanation for the fast and accurate conduction of the depolarizing wave along the 4 mm long and 80–130 μm wide heart of *T. molitor*, we propose an alternative model.

The differing conduction velocities of the wave of depolarization in the heart and the aorta was one of the challenges for the new model. The conduction velocity along the heart is approximately 10 times faster than the conduction velocity along the aorta, which consists only of ordinary muscle fibres. This indicated that another mechanism should be involved in conduction along the heart that is much faster than the conduction mechanism along ordinary aortic muscle fibres.

Such fast and accurate conduction velocities cannot be achieved by a series of pacemakers connected *via* a large number of gap junctions, as previously suggested. This has also been discussed by Miller and Usherwood (1971), who reported that communication through electrical junctions between the heart muscle fibres would be much too slow to coordinate the electrical activity and contraction of the many heart chambers in the cockroach.

Thus, we have replaced the many pacemaker myocardial cells spread along the heart with a single long pacemaker cell (or cells) that runs along the heart (Fig. 8B), but not in the aorta. This could be a differentiated muscle fibre, such as those found in vertebrate sinoatrial node, that has pacemaker properties. This pacemaker fibre (or fibres) must have an unusually high conduction velocity compared with the ordinary aortic muscle fibres. Similarly, it is well known that pacemaker cells in the sinoatrial node of mammals, for example, have high resting potentials that lead to high levels of excitability (Katz, 1992).

Our results indicate that every point along this differentiated muscle fibre has the potential to produce pacemaker activity. This is supported by our electrophysiological data, which showed not only that small fragments of the heart continued to contract rhythmically when isolated but also that they had the same intrinsic frequency. This clearly indicated that the isolated parts of the heart did not contain individual pacemaker cells coordinated by electrical synapses, as some have suggested, but are fragments of the same pacemaker cell.

The possible arrangement of the differentiated pacemaker fibres

At this point, it is appropriate to ask whether there are any cellular formations in the insect heart that could play the role of the differentiated muscle fibres described here. There are no data for the heart of *T. molitor*, but some speculations can be made on the basis of similar studies in other insects. Histological studies in moths and cockroaches have shown that the wall of the dorsal vessel is contractile and composed of a single layer of striated muscles arranged in an interlacing network of fibres running longitudinally and radially (McCann, 1963). The dorsal vessel of *Rhodnius prolixus*

consists of spirally arranged striated muscle fibres and possesses two rows of ventrally arranged longitudinal fibres extending the length of the abdomen. Embedded in the intima along each lateral side of the dorsal vessel are two large groups of endocardial cells extending the length of the dorsal vessel (Chiang et al., 1991). The situation is clearer in *Drosophila melanogaster*, in which the heart of adults and early pupae consists of a tube of circular striated muscle fibres one cell thick. A layer of longitudinal striated muscle fibres also occurs on the ventral surface of the heart (Curtis et al., 1999). Some of these longitudinal muscle fibres could be the differentiated muscle fibres with the pacemaker properties suggested here for the heart of *T. molitor*.

The directional depolarization

The results have shown that, in the intact differentiated muscle fibre, every point on its membrane has the potential to produce pacemaker activity but is driven by the pacemaker potential generated at the most posterior end of the cell (directional depolarization). When the heart was cut into two between the fourth and fifth cardiac segments, for example, the fourth segment became the posterior end, which now drove the rest of the heart with only minor changes in the heart rate and, therefore, the input driving the aorta. The situation was similar even when 90% of the heart was removed. Only when the last (most anterior) part of the heart was removed did electrical activity in the aorta cease. This indicates electrical coupling (a gap junction) between these differentiated muscle fibres in the heart and the ordinary muscle fibres of the aorta.

The directional depolarization observed in the heart of *T. molitor* is completely different from that in the vertebrate heart, in which there are a variety of pacemakers. This is supported by our results, which clearly indicated that, after the insect heart had been sectioned into two pieces, both parts could generate action potentials with the same frequency. We suggest that this is because the action potentials are generated by the same pacemaker cell now divided into two parts. In contrast, in vertebrate hearts with more than one pacemaker, the fastest pacemaker drives the whole heart.

The directional depolarisation of the pacemaker cells is vital to the functioning of the dorsal vessel as a peristaltic pump. The wall of the aorta is composed of a single layer of striated muscles arranged longitudinally and radially. Thus, when the wave of depolarization is generated in the sixth and last cardiac segment, it creates a local contraction of the radial muscles for 0.690 s, the mean duration of the depolarization at 60 beats min^{-1} . The contraction of the radial muscle fibres reduces the volume of this region of the heart, pushing haemolymph to the next (the fifth) cardiac segment. In the meantime, the wave of depolarization, which is transmitted with a velocity of 0.014 m s^{-1} , has already reached the fifth cardiac segment and produces a contraction of the local radial muscle fibres of this segment. This is repeated along the heart and the aorta. Thus, the heart of *T. molitor*, at 60 beats min^{-1} , acts as a peristaltic pump that transfers haemolymph from the posterior end of the abdomen to the head in less than a second.

The possible role of differentiated pacemaker cells during development

The fast recovery of the physiological properties of the heart pacemaker cells described above could be very important for the life cycle of *T. molitor*, and possibly for most holometabolous insects, during development. Drastic changes in the morphology and physiology not only of the dorsal vessel and the alary muscles but also the whole abdomen (Kalogianni et al., 1989) take place during metamorphosis. For example, in the larval stage of *T. molitor*, there are nine cardiac segments associated with nine pairs of fine alary muscle bundles. In the adult, this is reduced to six cardiac segments with six pairs of alary muscles, together with considerable changes in their morphology and physiology. As a simple example, in larvae, the aorta maintains its autorhythmicity even after separation from the heart; even fragments of the aorta can continue to contract rhythmically for hours (G. Theophilidis and T. Markou, unpublished observations). It is clear that, during histolysis at the pupal stage, certain cardiac segments must be removed and replaced, beginning with the last cardiac segments, as our results indicate. In other insects, the mononucleated larval heart undergoes autolysis during pupal life, and the newly formed imaginal cells are multinucleated (Tadkowski and McCann, 1980). Removal or even fusion of one, two or three cardiac segments during the pupal stage would cause cessation of heart activity, with pumping of the dorsal vessel stopping completely if there were only a primary pacemaker located in the posterior region of the heart. However, preliminary *in vitro* studies that we have conducted and the experiments of Tartes and Kuusik (1994) reveal a clear regularity in heart pulsation in the pupal stage of *T. molitor*, although the heart pulsation pattern changes during pupal development. In young pupae, the heart pumps weakly, in mid-pupae the systolic amplitude increases and in older pupae there is a low-frequency heart pulsation (Tartes and Kuusik, 1994). We believe that, because of the unusual ability of the pacemaker fibre (or fibres) to recover quickly following injury, to maintain its pacemaker action, the heart of the holometabolous insect *T. molitor* can maintain its function during the larval to adult transition. We speculate that this 'simple' type of pacemaker organization is a possible solution that enables the survival of holometabolous insects through metamorphosis.

References

- Ballard, R. C. and Hall, M. S.** (1969). A suction-electrode study of the house fly heart. *Ann. Ent. Soc. Am.* **62**, 375–379.
- Bruen, J. P. and Ballard, R. C.** (1970). An intracellular study of the myocardial cells of the flesh fly, *Sarcophaga bullata*. *Comp. Biochem. Physiol.* **32**, 227–236.
- Chapman, R. F.** (1975). *The Insects: Structure and Function*. London: The English Universities Press Ltd.
- Chiang, R. G., Chiang, J. A. and Davey, K. G.** (1991). Morphology of the dorsal vessel in the abdomen of the blood-feeding insect *Rhodnius prolixus*. *J. Morph.* **204**, 9–23.
- Consoulas, C. and Theophilidis, G.** (1992). Anatomy, innervation and motor control of the abdominal dorsal muscles of *Decticus albironis* (Orthoptera). *J. Insect Physiol.* **38**, 997–1010.
- Curtis, N. J., Ringo, J. M. and Dowse, H. B.** (1999). Morphology of the pupal heart, adult heart and associated tissues in the fruit fly, *Drosophila melanogaster*. *J. Morph.* **240**, 225–235.
- Dowse, H., Ringo, J., Power, J., Johnson, E., Kinney, K. and White, L.** (1995). A congenital heart defect in *Drosophila* caused by an action-potential mutation. *J. Neurogenet.* **10**, 153–168.
- Gu, G.-G. and Singh, S.** (1995). Pharmacological analysis of heartbeat in *Drosophila*. *J. Neurobiol.* **28**, 269–280.
- Johnson, E., Ringo, J., Bray, N. and Dowse, H.** (1998). Genetic and pharmacological identification of ion channels central to the *Drosophila* cardiac pacemaker. *J. Neurogenet.* **12**, 1–24.
- Jones, J. C.** (1954). The heart and associated tissues of *Anopheles quadrimaculatus* Say (Diptera Culicidae). *J. Morph.* **94**, 71–123.
- Kalogianni, E., Consoulas, C. and Theophilidis, G.** (1989). Anatomy and innervation of the abdominal segmental muscles in larvae and adult *Tenebrio molitor* (Coleoptera). *J. Morph.* **202**, 1–9.
- Kalogianni, E. and Theophilidis, G.** (1994). Motor innervation of the oviduct and central generation of the oviductal contractions in two orthopteran species (*Calliptamus* sp. and *Decticus albifrons*). *J. Exp. Biol.* **198**, 507–520.
- Katz, A. M.** (1992). *Physiology of the Heart*, second edition. New York: Raven Press.
- McCann, F. V.** (1962). Electrical activity in single myocardial cells of *Limulus polyphemus*. *Science* **137**, 340–341.
- McCann, F. V.** (1963). Electrophysiology of an insect heart. *J. Gen. Physiol.* **46**, 803–821.
- McCann, F. V.** (1965). Unique properties of the myocardium. *Ann. N.Y. Acad. Sci.* **127**, 84–99.
- McCann, F. V.** (1969). The insect heart as a model for electrophysiological studies. *Exp. Physiol. Biochem.* **2**, 59–88.
- McCann, F. V.** (1971). Calcium action potentials in insect myocardial fibers. *Comp. Biochem. Physiol.* **40A**, 353–357.
- Miller, T. A.** (1969). Initiation of activity in the cockroach heart. *Experientia* **15** (Suppl.), 206–218.
- Miller, T. A.** (1974). Electrophysiology of an insect heart of the American cockroach. In *The Physiology of Insecta*, second edition, vol. 4 (ed. M. Rockstein), pp. 169–199. New York, London: Academic Press.
- Miller, T. A.** (1985). Intergument, respiration and circulation: Structure and physiology of the circulatory system. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 290–349. Oxford: Pergamon Press.
- Miller, T. A. and Metcalf, R. L.** (1968). Site of action of pharmacologically active compounds on the heart of *Periplaneta americana* L. *J. Insect Physiol.* **14**, 383–394.
- Miller, T. A. and Usherwood, P. N. R.** (1971). Studies of cardio-regulation in the cockroach *Periplaneta americana*. *J. Exp. Biol.* **54**, 329–348.
- Richter, M. and Hertel, W.** (1997). Contribution to physiology of the antenna-heart in *Periplaneta americana* (L.) (Blattodea: Blattellidae). *J. Insect Physiol.* **43**, 1015–1021.
- Rugendorff, A., Younossi-Hartenstein, A. and Hartenstein, V.** (1994). Embryonic origin and differentiation of the *Drosophila* heart. *Roux's Arch. Dev. Biol.* **203**, 266–280.
- Sanger, J. W. and McCann, F. V.** (1968). Ultrastructure of the myocardium of the moth, *Hyalophora cecropia*. *J. Insect Physiol.* **14**, 1105–1111.
- Smith, N. A.** (1969). Observations on the neural rhythmicity in the cockroach. *Experientia* **15** (Suppl.), 200–205.

- Sperelakis, N. and Banks, O. R.** (1993). *Physiology*, first edition. Boston, Toronto, London: Little, Brown & Company.
- Tadkowski, T. M. and McCann, F. V.** (1980). Ultrastructure and electrical activity in developing heart cells (insect). *Devl. Biol.* **74**, 378–400.
- Tartes, U. and Kuusik, A.** (1994). Periodic muscular activity and its possible function in pupae *Tenebrio molitor*. *Physiol. Ent.* **19**, 216–222.
- Tublitz, N.** (1998). Insects cardioactive neuropeptides: Peptidergic modulation of the intrinsic rhythm of an insect heart is mediated by inositol 1,4,5-trisphosphate. *J. Neurosci.* **8**, 4394–4699.
- Wigglesworth, V. B.** (1974). *The Principles of Insect Physiology*, seventh edition, pp. 411–457. New York: John Wiley & Sons.
- Wu, C.-F. and Ganetzky, B.** (1980). Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila melanogaster*. *Nature* **286**, 814–816.
- Wu, C.-F., Ganetzky, B., Jan, L., Jan, Y. and Benzer, S.** (1978). A *Drosophila* mutant with a temperature-sensitive block in nerve conduction. *Proc. Natl. Acad. Sci. USA* **75**, 4047–4051.
- Yeager, J. F.** (1939). Electrical stimulation of isolated heart preparation from *Periplaneta americana*. *J. Agric. Res.* **59**, 121–132.