

# Neuronal and neurohormonal control of the heart in the stomatopod crustacean, *Squilla oratoria*

Hiroshi Ando<sup>1</sup> and Kiyoaki Kuwasawa<sup>2,\*</sup>

<sup>1</sup>Department of Oral Physiology, Matsumoto Dental University School of Dentistry, Shiojiri 399-0781, Japan and

<sup>2</sup>Neurobiology Laboratory, Faculty of Science, Okayama University of Science, Ridai-cho 1-1, Okayama 700-0005, Japan

\*Author for correspondence (e-mail: kuwasawa-kiyoaki@das.ous.ac.jp)

Accepted 6 September 2004

## Summary

The heart of *Squilla oratoria* contains a cardiac ganglion that consists of 15 intrinsic neurons, supplied by a pair of inhibitory nerves and two pairs of excitatory nerves, arising from the central nervous system. These comprise the extrinsic cardiac innervation. The paired cardio-inhibitor (CI) nerves run out in the 10th pair of nerve roots emerging from the subesophageal ganglion (SEG). The cell bodies of the CI neurons are found in the hemisphere of the 1st segment of the SEG contralateral to the nerve roots in which the CI axons emerge. The two pairs of 1st and 2nd cardio-accelerator (CA1 and CA2) nerves run out in the 16th and 19th pairs of nerve roots of the SEG. The cell bodies of the CA1 and CA2 neurons are found in the hemispheres of the 3rd and 4th segments of the SEG ipsilateral to the nerve roots in which the CA1 and CA2 axons are found.

The heartbeat was activated by application of glutamate, serotonin, dopamine, octopamine or acetylcholine, which were applied to the heart by perfusion into an organ bath. Joro-spider toxin (JSTX) blocked myocardial excitatory junctional potentials

evoked by the cardiac ganglion. Neuronal cell bodies and processes in the heart were examined using immunocytochemical techniques. All 15 neurons of the cardiac ganglion showed glutamate-like immunoreactivity. Glutamate may be a neurotransmitter of the cardiac ganglion neurons.

JSTX also blocked cardiac acceleration by activation of CA1 and CA2 axons. CA1 and CA2 axons showed glutamate-like immunoreactivity. It is likely that glutamate is a neurotransmitter for the cardio-acceleratory neurons.

The heartbeat was inhibited by application of  $\gamma$ -aminobutyric acid (GABA). Cardiac inhibition induced by activation of CI axons was blocked by picrotoxin. CI axons showed GABA-like immunoreactivity. These results may support the identification of GABA as an extrinsic inhibitory neurotransmitter.

**Key words:** cardioregulatory neuron, cardiac ganglion neuron, neurotransmitter, stomatopod, *Squilla oratoria*, immunocytochemistry, GABA, glutamate.

## Introduction

Alexandrowicz (1934) first showed that three pairs of central cardioregulatory nerves ( $\alpha$ ,  $\beta$  and  $\gamma$ ) control the heart of the stomatopod *Squilla mantis*. Of these,  $\beta$  and  $\gamma$  are most probably nerves originating from the 3rd and 4th segments of 'the large thoracic ganglionic mass', while  $\alpha$  is a nerve originating from a more anterior segment of the ganglionic mass. He proposed that the  $\alpha$  nerve was a cardio-inhibitor while the  $\beta$  and  $\gamma$  nerves were cardio-accelerators. The functions of  $\alpha$ ,  $\beta$  and  $\gamma$  nerves in control of the heart were verified by means of intracellular recording from neuronal cell bodies in the cardiac ganglion of *Squilla oratoria* (Watanabe et al., 1968, 1969). It is known that the heartbeat of stomatopods is triggered by the cardiac ganglion, located on the outer surface of the heart (reviewed by Maynard, 1960). Neither the anatomical studies of Alexandrowicz (1934) nor the functional studies of Watanabe

et al. (1968, 1969), have been followed by more detailed studies of stomatopod neuroanatomy, and until the present study, a detailed description of the anatomy of the central ganglia and of the ganglionic nerve roots from which the cardioregulatory axons emerge has not been published, nor have their cell bodies been identified in the central ganglia.

The neurotransmitter candidates for the intrinsic and extrinsic heart neurons have been identified in a few species within the sub-class Malacostraca from the results of pharmacological and immunocytochemical studies. Dealing with the decapods, Yazawa and Kuwasawa (1994) proposed that  $\gamma$ -amino-butyric acid (GABA) and dopamine (DA) are the extrinsic neurotransmitters of the cardio-inhibitory and cardio-acceleratory nerves, respectively, in the hermit crab *Aniculus aniculus*. They also proposed that acetylcholine (ACh) and DA

are the intrinsic neurotransmitters of the small and large neurons, respectively, in the cardiac ganglion of that species (Yazawa and Kuwasawa, 1992). On the other hand, glutamate (Glu) has been proposed for the neurotransmitter of both of the small and large neurons in the cardiac ganglion of the lobster *Panulirus argus* (Delgado et al., 2000). In isopods, it has been proposed that Glu is the intrinsic excitatory neurotransmitter released by the motoneurons of the cardiac ganglion of *Bathynomus doederleini* (Yazawa et al., 1998) and *Ligia exotica* (Sakurai et al., 1998). Furthermore, GABA has been proposed as the extrinsic cardio-inhibitory neurotransmitter, while ACh has been proposed as the extrinsic cardio-acceleratory neurotransmitter in *B. doederleini* (Tanaka et al., 1992).

In stomatopods, epinephrine (E), norepinephrine (NE), and ACh have cardioexcitatory effects on the heart of *S. mantis* (Alexandrowicz and Carlisle, 1953; Florey and Rathmayer, 1990). Additionally, pharmacological experiments indicate that GABA may be a neurotransmitter of the extrinsic cardio-inhibitor nerves of *S. oratoria* (Watanabe et al., 1968). However, candidate neurotransmitters of the cardiac ganglion neurons and cardio-accelerator nerves have not yet been proposed for the stomatopod (recently reviewed by Cooke, 2002).

In this study, we describe the central ganglia and the nerve roots from which the cardioregulatory axons emerge, and identify their cell bodies in the ganglia. Furthermore, we identify neurotransmitter candidates for all the cardioregulatory neurons and neurons of the cardiac ganglion, from the results of electrophysiological, pharmacological and immunocytochemical studies. Preliminary reports have appeared elsewhere in abstract form (Ando and Kuwasawa, 1993; Ando and Kuwasawa, 1994; Ando et al., 1995).

### Materials and methods

The stomatopods, *Squilla oratoria* De Haan used in this study were about 10–15 cm in body length and about 20 g in mass. Specimens were collected in fishing trawl nets in Tokyo Bay and kept for a few days to several months in a laboratory aquarium maintained at 13–15°C.

#### Neuroanatomy

Before dissection, the animals were anesthetized by injection of an isotonic  $\text{MgCl}_2$  ( $0.36 \text{ mol l}^{-1}$ ) solution, through a syringe inserted into the thorax. After anesthetization, they were pinned to the Silpot (Dow Corning, Kanagawa, Japan)-lined bottom of a chamber filled with cold seawater, kept in a refrigerator. The carapace in the cephalo-thorax was carefully peeled off to expose the terminal region of the cardioregulatory nerves on the heart inside the pericardium. The peripheral cardioregulatory nerves were stained by means of a vital staining technique, in Methylene Blue filtered seawater (SW). The stained preparations were fixed in a 4% ammonium molybdate–SW solution overnight and washed in running tapwater for several hours (Alexandrowicz, 1932). Then, the

fixed preparations were stained in a Methylene Blue–tapwater solution (Kihara and Kuwasawa, 1984), and dissection was continued to trace the cardioregulatory nerves up to the central ganglia.

According to their functions, as elucidated by physiological experiments (see Electrophysiology section below), we refer to the nerves named  $\alpha$ ,  $\beta$  and  $\gamma$  by Alexandrowicz (1934) as, respectively, the cardio-inhibitor (CI) nerve, and the 1st and 2nd cardio-accelerator (CA1 and CA2) nerves. Those are terms that have been commonly used in a variety of crustacean species: in the decapods (Maynard, 1953; Yazawa and Kuwasawa, 1984) and in the isopods (Kihara and Kuwasawa, 1984; Sakurai and Yamagishi, 1998).

#### Electrophysiology

After the heart and cardioregulatory nerves were exposed, the nerves were cut in the pericardial cavity. The isolated heart was pinned dorsal side up to a Silpot-lined experimental bath (3.5 ml). The distal cut-stumps of the nerves, extending to the heart, were introduced one by one into a glass capillary suction electrode containing a Ag–AgCl wire, used to apply stimulus pulses to the nerve. Heartbeat of the whole heart was monitored using either a mechanogram, with a strain gauge transducer (TB-611T Nihon Kohden, Tokyo, Japan) or an electrocardiogram (ECG), which was recorded from the outer surface of the heart with a glass capillary suction electrode.

Myocardial intracellular potentials were recorded from the isolated heart, which was opened by longitudinal incision of the ventral wall of the heart. After incision, the heart was spread out and then pinned dorsal side up to a Silpot-lined experimental bath (1.5 ml). Intracellular potentials were recorded with a conventional glass capillary microelectrode filled with  $3 \text{ mol l}^{-1}$  KCl (tip resistance, 20–35 M $\Omega$ ). Excitatory junctional potentials (EJPs) in myocardial cells were evoked by electrical stimuli applied to the cardiac ganglion at the cut stump of the trunk of the ganglion. Preparations were routinely perfused with *Squilla* saline (in mmol  $\text{l}^{-1}$ : NaCl, 450; KCl, 15;  $\text{CaCl}_2$ , 10;  $\text{MgCl}_2$ , 20; Hepes, 5; pH 7.8) by gravity-feeding through a cannula at the rate of 3–4 ml  $\text{min}^{-1}$ . Saline was suctioned for removal. The saline was modified from the saline of Watanabe et al. (1967). The perfusate was maintained at 17–20°C by a cooling device (EC-201, Scinics, Tokyo, Japan).

#### Application of agents

The following agents were used: acetylcholine chloride (ACh), atropine sulfate monohydrate, dopamine hydrochloride (DA),  $\gamma$ -amino-*N*-butyric acid (GABA), sodium L-glutamate monohydrate, histamine dihydrochloride, joro spider toxin 3 (JSTX), L-noradrenaline bitartrate, picrotoxin (PTX), serotonin–creatinine sulfate, serotonin (5-HT), D-tubocurarine hydrochloride (Wako Pure Chemical Industries, Osaka, Japan), adrenaline bitartrate, chlorpromazine hydrochloride, octopamine hydrochloride (Sigma Chemicals, St Louis, MO, USA), phentolamine mesylate, Regitin (Ciba-Geigy, Basle, Switzerland).

The salines containing the agents were applied to preparations when the cock of a three-way valve was turned in the perfusion line, except for JSTX, when saline containing the agent was added to the experimental bath by a pipette.

#### *Resin sections for photomicroscopic observation*

Pieces of nerve, isolated from the CI, CA1 and CA2, were pre-fixed in a 3% (w/v) glutaraldehyde solution and post-fixed in a 0.1% (w/v) osmium solution. After washing in the buffer solution and dehydrating in a graded ethanol series, they were embedded in Quetol-812 (Nissin EM, Tokyo, Japan). Sections were cut at 1  $\mu\text{m}$  and stained with the mixed 1% (w/v) Methylene Blue and 0.1% (w/v) Azur II solutions. They were observed under a microscope and photographed (BX-51, Olympus, Tokyo, Japan).

#### *Back-filling of the cardioregulatory neurons with $\text{Co}^{2+}$ and $\text{Ni}^{2+}$ ions*

In order to locate the cell bodies of the CI, CA1 and CA2 neurons in the central nervous system (CNS), a proximal cut-stump of the cardioregulatory nerve was introduced into a glass capillary filled with a mixed solution of 1  $\text{mol l}^{-1}$   $\text{CoCl}_2$  and 1  $\text{mol l}^{-1}$   $\text{NiCl}_2$ . The preparation was incubated for 4–8 days at 4°C, and then rinsed with SW. Some drops of a saturated Rubenic Acid–ethanol solution were added to precipitate a sulfate compound (Quicke and Brace, 1979). The stained preparation was fixed with 4% (w/v) formaldehyde, dehydrated with a graded ethanol series, and cleared with methyl salicylate. The preparation was observed under a microscope and photographed (BX-51, Olympus).

#### *Immunocytochemistry*

We examined immunoreactivity of neural processes in the heart and extrinsic nerves against four kinds of antibodies: anti-GABA, anti-glutamate, anti-serotonin and anti-histamine. In immunocytochemistry using anti-GABA and anti-glutamate, preparations were processed as described for the isopod by F.-Tsukamoto and Kuwasawa (2003). For controls, preparations were processed in the same manner but without treatment with the primary antibodies. For each antibody, no immunoreactivity was detected in the control preparations. The preparations were observed under a microscope and photographed (BX-51, Olympus).

##### *1. Treatment with anti-GABA antibodies*

To locate GABA-like immunoreactive neural processes, specimens were fixed with a solution of 4% (w/v) paraformaldehyde–0.1% glutaraldehyde in 0.1  $\text{mol l}^{-1}$  phosphate buffer (pH 7.2) containing 15% (w/v) sucrose for 2–3 h at 4°C. After fixation they were rinsed in a 0.1  $\text{mol l}^{-1}$  phosphate buffer solution containing 15% (w/v) sucrose over 1 day at 4°C. The specimens were embedded in paraffin and sectioned serially at 10  $\mu\text{m}$ . The sections were dried on the slides, deparaffinized, rehydrated and immersed in distilled water (DW). In order to eliminate intrinsic peroxidase

activity in the sectioned tissues, the slides were treated with 0.3% (w/v)  $\text{H}_2\text{O}_2$  in DW for 30 min at room temperature. They were incubated with a 0.1  $\text{mol l}^{-1}$  phosphate buffer containing 0.1% (w/v) Triton X-100 (pH 7.2) (0.1% PBT) for 15 min, then with the primary antibody (rabbit anti-GABA; cat. no. 20094, Incstar, Stillwater, MN, USA), diluted 1:2000 in a 0.1% PBT for 24 h and rinsed in a 0.1% PBT for 1 h. The specimens were treated with the secondary antibody (goat anti-rabbit IgG; Sigma), diluted 1:200 in a 0.1% PBT for 1.5–2 h at room temperature and rinsed in a 0.1% PBT for 1 h. They were treated with the third antibody [rabbit peroxidase–antiperoxidase (PAP) complex; Sigma], diluted 1:200 in a 0.1% PBT for 2 h and rinsed in a 0.1% PBT for 1 h. 0.03% (w/v) 3,3'-diaminobenzidine-tetrahydrochloride (DAB) in 0.05  $\text{mol l}^{-1}$  Tris buffer (pH 7.6) containing 0.006% (w/v)  $\text{H}_2\text{O}_2$  was applied for about 10 min at room temperature in dark. The peroxidase reaction was stopped by transferring the slides to DW. The sections were counterstained with Methyl Green, dehydrated in a graded ethanol series, cleared in xylene and mounted in Bioleite (Oken Shoji, Tokyo, Japan).

For whole-mount preparations, fixed specimens were immersed in 0.2% (w/v) collagenase in SW, rinsed in 0.3% PBT, and immersed in 1% PBT overnight. They were incubated with the primary antibody diluted 1:1000 in a 0.3% PBT for 48 h, with the secondary antibody for 2–4 h and further with PAP complex for 2–4 h. After each incubation they were rinsed in 0.3% PBT for 1 h. The specimens were stained with DAB solutions, dehydrated with a graded ethanol series, cleared in methyl salicylate and then in xylene and mounted on glass slides in Bioleite.

##### *2. Treatment with anti-glutamate antibodies*

Specimens were treated as described for GABA immunocytochemistry before treatment with the antibodies. Specimens were treated with the primary antibody (mouse anti-glutamate; cat. no. 22523, Incstar or cat. no. G9282, Sigma) diluted 1:1000 (Incstar) or 1:20000 (Sigma) in a 0.1% PBT. The specimens were treated with the secondary antibody (goat anti-mouse IgG; Sigma), diluted 1:200 in a 0.1% PBT and with the third antibody (mouse PAP complex; Sigma), diluted 1:200 in a 0.1% PBT. Then the specimens were processed and examined as described above.

##### *3. Treatment with anti-serotonin antibodies*

To locate serotonin-like immunoreactive neural cells and processes, specimens were fixed with a solution of 4% (w/v) paraformaldehyde in 0.1  $\text{mol l}^{-1}$  phosphate buffer containing 15% (w/v) sucrose (pH 6.5) for 30 min at room temperature, followed by a 4% (w/v) paraformaldehyde in 0.1  $\text{mol l}^{-1}$  sodium tetraborate buffer containing 15% (w/v) sucrose (pH 9.0) for 2.5–3 h at 4°C. The specimens were treated with the primary antibody (rabbit anti-serotonin; cat. no. 2008, Incstar), diluted 1:2000 in a 0.1% PBT for 24 h and rinsed in a 0.1% PBT. Then, the specimens were processed and examined as described above.

#### 4. Treatment with anti-histamine antibodies

To locate histamine-like immunoreactive neural cells and processes, specimens were fixed with a solution of 4% (w/v) ethyl-dimethylcarbodiimide in 0.1 mol l<sup>-1</sup> phosphate buffer (pH 7.3) for 60 min at 4°C, followed by a 4% (w/v) paraformaldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer containing 15% sucrose (pH 7.2) overnight at 4°C. The specimens were treated with the primary antibody (rabbit anti-histamine; cat. no. 22939, Incstar), diluted 1:1000 in a 0.1% PBT for 24 h and rinsed in a 0.1% PBT. Then, the specimens were processed and examined as described above.

## Results

### Neuroanatomy

In stomatopods, the cephalon and the 1st to 4th thoracic segments are fused to form the cephalo-thorax, which is

followed by the 5th to 8th thoracic segments, the 1st to 6th abdominal segments and the telson (McLaughlin, 1980). The topographical relationship between the exoskeleton, the heart and CNS in *S. oratoria* is shown in Fig. 1. The heart lies over a range from the 2nd maxilla in the cephalo-thorax to the 5th abdominal segment. The anterior artery (AA), 15 pairs of the lateral arteries (LA1-15) and the posterior artery (PA) arise from the heart. The subesophageal ganglion (SEG) is connected with the cerebral ganglion by the circumesophageal nerve ring and is followed by the thoracic ganglia and abdominal ganglia.

The cardiac ganglion (CG), which lies on the outer surface of the heart, consists of 13–16 ganglion cells in stomatopods (Alexandrowicz, 1934; Irisawa and Irisawa, 1957; Brown, 1964a; Watanabe et al., 1967; Florey and Rathmayer, 1990). We used Methylene Blue staining techniques to determine the number of neurons in the CG of the present material. We

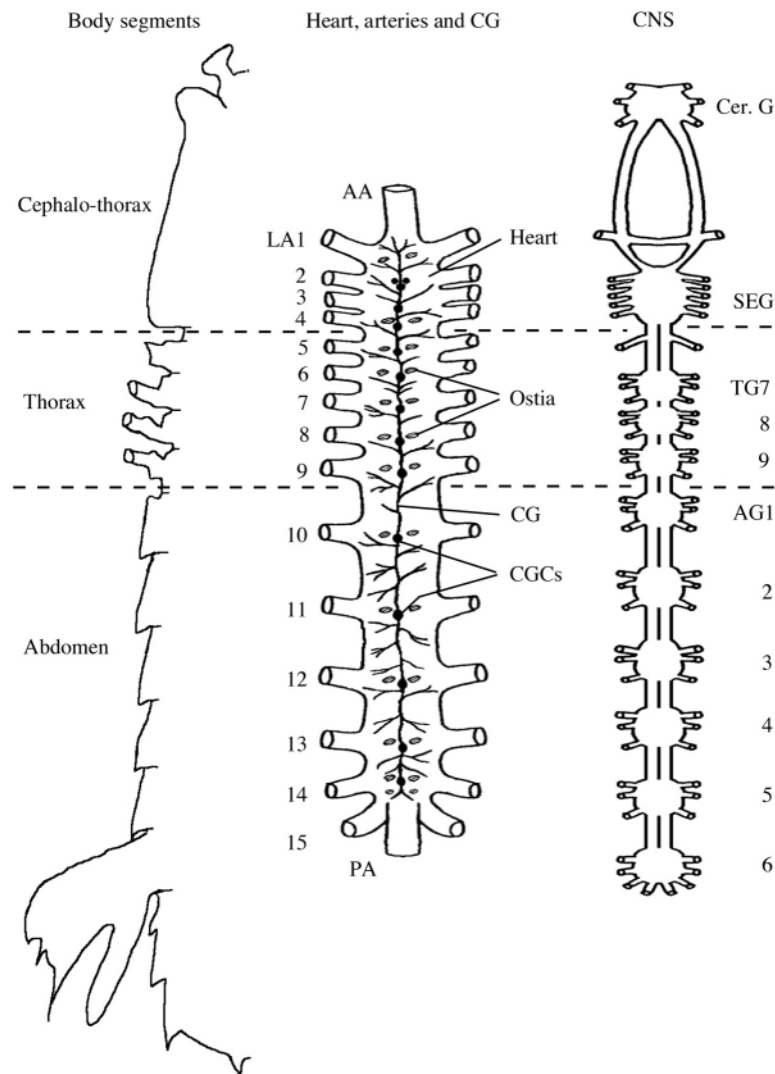


Fig. 1. Schematic drawing of the heart and bases of the arteries (dorsal view), and the CNS (ventral view) corresponding to the body segments. AA, anterior artery; AG1-6, 1st to 6th abdominal ganglia; Cer. G, cerebral ganglion; CG, cardiac ganglion; CGCs, cardiac ganglion cells; LA1-15, 1st to 15th lateral artery; PA, posterior artery; SEG, subesophageal ganglion; TG7-9, 7th to 9th thoracic ganglia.

counted 15 cell bodies in the area of the CG between the 2nd and 14th lateral arteries for more than 50 preparations, which were well stained with the dye. Three of the 15 cell bodies formed a cluster at the base of the 2nd arteries, and the other 12 cell bodies were individually located at each base of the

lateral arteries. The number of cell bodies coincides with that of pairs of the lateral arteries. Fig. 2 shows the cardiac ganglion cells in the CG in the anterior part of the heart. Three anterior cell bodies of the ganglion lie forming a cluster at the 2nd lateral artery.

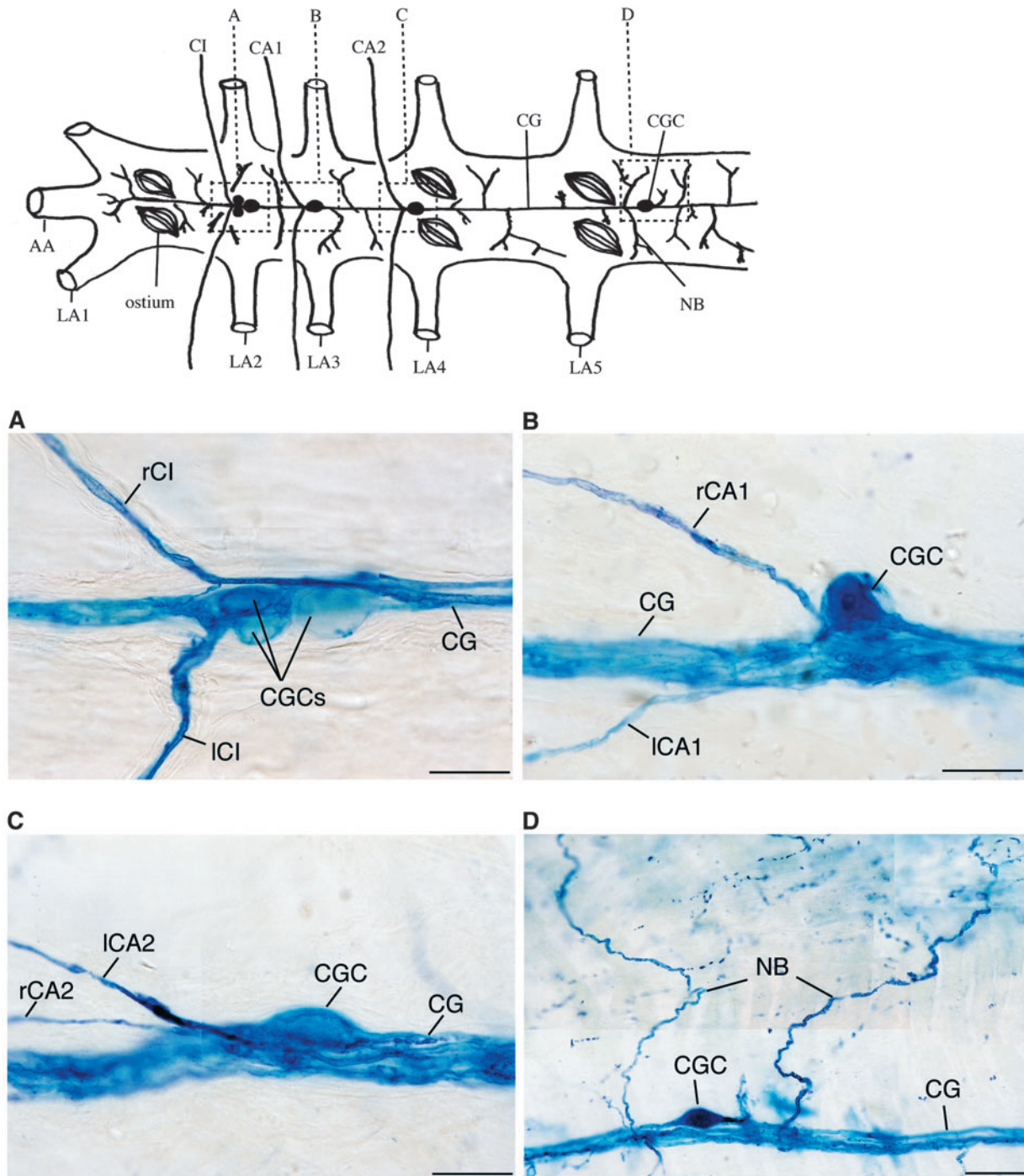


Fig. 2. (Top) Schematic drawing shows the cardiac ganglion and the right (r) and left (l) CI, CA1 and CA2 nerves joining the cardiac ganglion (CG) in the anterior part of the heart. (A–D) Photographs of the the outlined areas in the top diagram, showing the cardiac ganglion and cardioregulatory nerves in the cardiac ganglion trunk (CGT) in the anterior region of the heart stained with Methylene Blue. Neural branch (NB) from the trunk of the CG extends to the myocardium. AA, anterior artery; LA1 to LA5, The 1st to 5th lateral arteries. Scale bars, 50  $\mu$ m.

Three pairs of cardioregulatory nerves join the CG at the level of the origins of the 2nd, 3rd and 4th lateral arteries in the cephalo-thorax, for CI, CA1 and CA2 nerves, respectively (Fig. 2A–C).

Anatomically, we traced the nerves all the way to the central nervous system from which they arose, and located their origins in the SEG. We decided that the CI, CA1 and CA2 nerves emerged from, respectively, the 10th, 16th and 19th nerve roots (10th NR, 16th NR and 19th NR) of the SEG (Fig. 3A). In order to verify these findings, we observed the effects of stimulation of their roots on heart beat.

When the 10th NR was electrically stimulated at a distal cut-stump of the nerve, heart rate decreased (Fig. 3B). The effect was intensified with increased stimulus frequency (Fig. 3C). By contrast, when either the 16th or 19th NR was electrically stimulated at a distal cut-stump of each nerve, heart rate and amplitude of action potentials increased (Fig. 3B). The

acceleratory effect was intensified with increased stimulus frequency (Fig. 3C). Fig. 3C shows the effects of stimulation of the nerve roots on heart rate. Heart rate in controls was  $33.2 \pm 8.9$  for the 10th NR,  $30.0 \pm 7.7$  for the 16th NR and  $26.6 \pm 6.1$  for the 19th NR (mean  $\pm$  s.d.;  $N=7$  for the 10th NR,  $N=9$  for the 16th NR,  $N=10$  for the 19th NR). The increased amplitude of action potential, during the stimulations, may indicate an increase of contraction force of the heart. Thus, the anatomical results described above were confirmed by these results.

In order to examine the axonal composition of the cardioregulatory nerves, extracellular impulses were recorded from the nerves. Only one kind of orthodromic (Fig. 4A orth) or antidromic unit impulse (Fig. 4A ant) was recorded for CI, CA1 and CA2. Additional impulse units never appeared, even when stimulated at higher intensity. These results were confirmed in seven preparations. Resin cross sections obtained

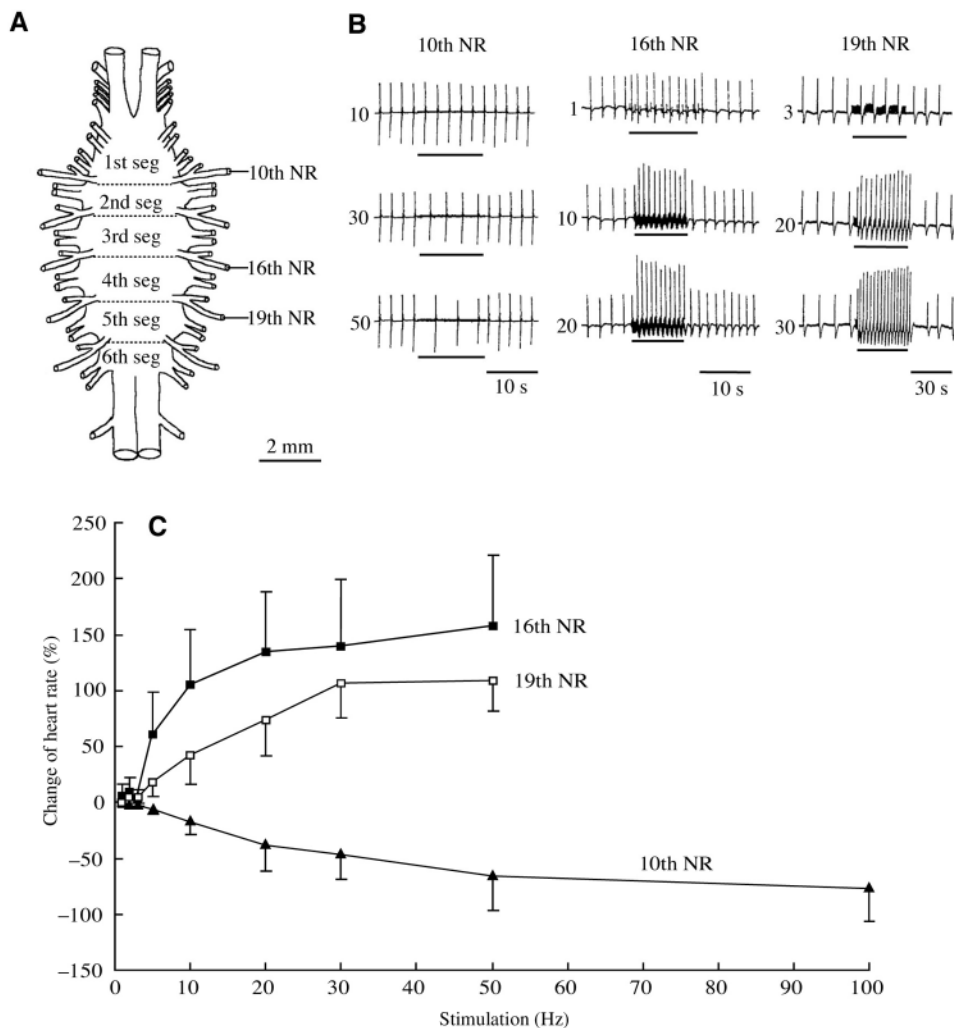


Fig. 3. (A) Schematic drawing of the SEG. seg, segments; NR, nerve roots of SEG. (B) Effects of stimulation of the 10th, 16th and 19th NR on ECG. The roots were anatomically determined to contain, respectively, CI, CA1 or CA2 nerves. The bar shows a period of repetitive stimulation. Stimulus frequencies (Hz) are shown at the beginning of the recordings. (C) Effects of stimulations of the 10th, 16th or 19th nerve root on heart rate. Values are means  $\pm$  s.d.;  $N=7$  for 10th NR,  $N=9$  for 16th NR,  $N=10$  for 19th NR.

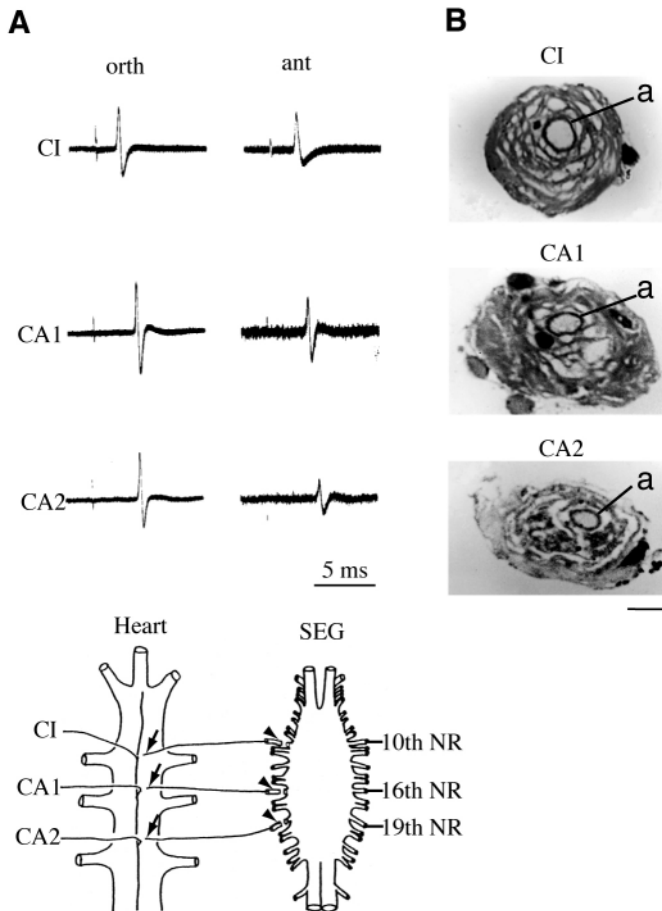


Fig. 4. (A) Orthodromically conducted impulses were recorded at the proximal cut-stump of the CI, CA1 and CA2 nerves on the heart (orth). The impulses were induced by stimulation of the nerve roots at the distal cut stumps of the 10th, 16th and 19th roots of the SEG, respectively. The sites of recording and stimulation of antidromically conducted impulses (ant) were contrary to those of orth. Five traces were superimposed in each recording. The diagrams below show stimulation and recording on the cutting sites for CI, CA1 and CA2 (arrows) and 10th, 16th and 19th nerve roots (NR; arrowheads). The right and left sides of the SEG are reversed. (B) Micrographs of resin cross sections of the CI, CA1 and CA2 nerves. A single axon (a) is seen in each section. Scale bar, 10  $\mu$ m.

from the CI, CA1 and CA2 nerves were observed under a microscope (Fig. 4B), and showed that each of the CI, CA1 and CA2 nerves contains one axon (6–8  $\mu$ m in diameter) wrapped in the heavily stained perineurium (about 1  $\mu$ m in thickness), which is surrounded by epineurium (10–20  $\mu$ m in thickness), thus forming the nerves.

The axons were back-filled with  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$  ions at their proximal cut-stumps. The cell body (about 30  $\mu$ m in diameter) of the CI axon was found stained at a site near the midline on the side contralateral to the nerve root through which the CI axon emerged in the 1st SEG segment (Fig. 5). The cell body (about 20  $\mu$ m in diameter) of the CA1 axon was found stained at a site near the midline on the side ipsilateral to the nerve

root through which the CA1 axon emerged in the 3rd SEG segment. The cell body (20  $\mu$ m in diameter) of the CA2 axon was found stained at a site near the midline on the side ipsilateral to the nerve root through which the CA2 axon emerged in the 4th SEG segment.

#### *Effects of putative neurotransmitters and neurohormones*

In order to examine the effects on the heart of putative neurotransmitter substances known in crustaceans (Cooke and Sullivan, 1982), mechanograms of heartbeat were recorded from isolated hearts treated with the substances.

Effects of GABA and histamine on the heart are shown in Fig. 6. GABA ( $>10^{-6}$  mol  $\text{l}^{-1}$ ) decreased heart rate and contraction force depending on the dose (Fig. 6A). Similar results were reported by Watanabe et al. (1968) for GABA application to the heart of *S. oratoria*. Histamine (HA) ( $>10^{-6}$  mol  $\text{l}^{-1}$ ) also exerted inhibitory effects on the heart rate and contraction force (Fig. 6B). Picrotoxin, a GABAergic antagonist, at  $10^{-4}$  mol  $\text{l}^{-1}$ , completely blocked the cardiac inhibition induced by stimulation of the CI (Fig. 6C).

5-HT, DA, OA, ACh and Glu were applied to the isolated heart, and dose-response curves are shown in Fig. 7. 5-HT initially transiently decreased both heart rate and contraction force, and then increased both heart rate and contraction force (Fig. 7A). The effects of 5-HT on heart rate and contraction force increased in a dose-dependent manner. Threshold concentrations for 5-HT in both the first and second phases were between  $10^{-9}$  and  $10^{-8}$  mol  $\text{l}^{-1}$ . DA (Fig. 7B), OA (Fig. 7C), ACh (Fig. 7D) and Glu (Fig. 7E) increased heart rate and force. Threshold concentrations for both DA and OA were approximately  $10^{-8}$  mol  $\text{l}^{-1}$ , and those for both ACh and Glu around  $10^{-6}$  mol  $\text{l}^{-1}$ . Epinephrine (E) and norepinephrine (NE) showed weak excitatory effects compared to the effects induced by stimuli of 5-HT, DA and OA (data not shown).

Antagonists for various putative neurotransmitter substances were tested in order to predict the neurotransmitters of the extrinsic CA axons and cardiac ganglion neurons. The catecholaminergic blockers, chlorpromazine  $10^{-5}$  mol  $\text{l}^{-1}$  and phentolamine  $10^{-5}$  mol  $\text{l}^{-1}$ , did not antagonize the cardio-acceleratory effects of CA nerves and force of the myocardium beats induced by the cardiac ganglion. The cholinergic blockers, atropine  $10^{-4}$  mol  $\text{l}^{-1}$  and d-tubocurarine  $10^{-4}$  mol  $\text{l}^{-1}$ , were also ineffective on the acceleratory effects produced by stimulation of the CA nerves (data not shown). Three preparations were used for each of the agents.

JSTX ( $10^{-5}$  mol  $\text{l}^{-1}$ ), known as a glutamate antagonist (Kawai et al., 1982; Chiba and Tazaki, 1992; Sakurai et al., 1998; F.-Tsukamoto and Kuwasawa, 2003), blocked cardio-acceleratory effects of stimulation of the CA1 and CA2 axon (Fig. 8). Acceleratory effects of stimulation of CA1 or CA2 decreased to  $38.5 \pm 12.7\%$  of control value for CA1 and  $24.5 \pm 17.5\%$  for CA2 (means  $\pm$  S.D.,  $N=3$ ).

$5 \times 10^{-6}$  mol  $\text{l}^{-1}$  JSTX blocked EJPs in cardiac muscle, evoked by stimulation of the cardiac ganglion trunk (Fig. 9).

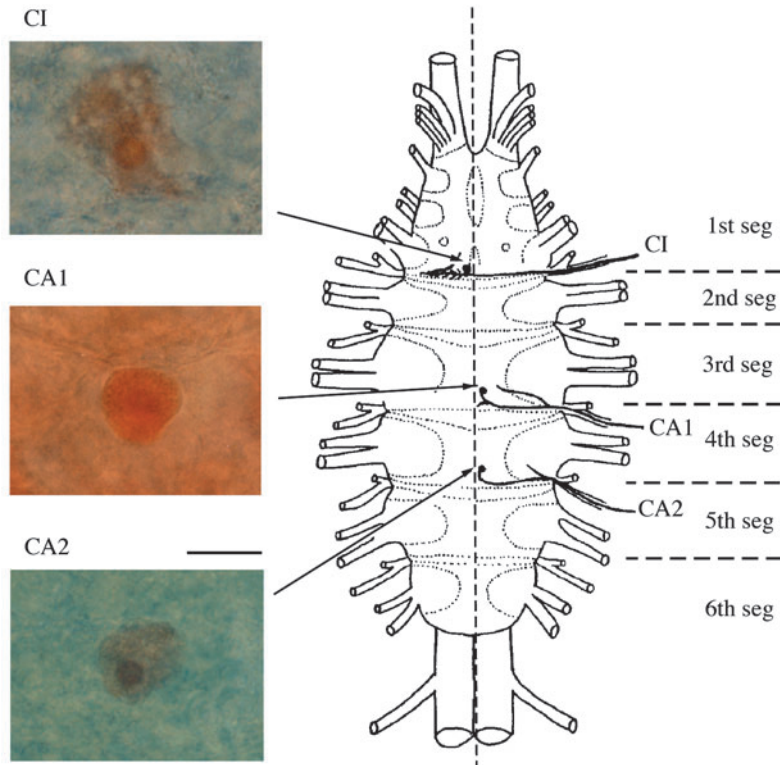


Fig. 5. Micrographs of the cell bodies of CI, CA1 and CA2 neurons stained by back-filling with  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$  ions. The schematic drawing (right) shows their locations in the CNS. The cell body of the CI neuron was located at a site near the midline on the side contralateral to the 10th nerve root containing the CI axon in the 1st segment. The cell bodies of the CA1 and CA2 neurons were located at sites, near the midline on the side ipsilateral to the 3rd nerve root in the 3rd and 4th segments, respectively. Scale bar, 20  $\mu\text{m}$ .

The amplitude of EJPs decreased to  $29.1 \pm 14.6\%$  of control values (mean  $\pm$  S.D.,  $N=9$ ). EJPs partially recovered at 3 h after washout of JSTX.

#### Immunocytochemistry

No immunoreactivity was detected in a negative control preparation of wholemount preparations or paraffin sections incubated without the primary anti-GABA and anti-glutamate antibodies.

#### GABA-like immunoreactivity

The CI axon showed GABA-like immunoreactivity to anti-GABA antibodies (Fig. 10A). An axon showing GABA-like immunoreactivity runs in the CG (Fig. 10B). Although many histamine-like immunoreactive neuronal processes, as well as GABA-like immunoreactive ones, were observed in the SEG, the CI nerves showed no histamine-like immunoreactivity to anti-histamine antibodies (data not shown).

#### Glutamate-like immunoreactivity

Fig. 11 shows glutamate-like immunoreactive neuronal processes, evoked in response to anti-glutamate antibodies. Both the CA1 and CA2 axons and the CG showed glutamate-like immunoreactivity (Fig. 11A). A glutamate-like immunoreactive cell body was observed in Fig. 10B. Glutamate-like immunoreactivity was shown in a major motor branch extending from the CG (Fig. 11C). Glutamate-like immunoreactivity was observed in all cells in the CG (data not shown). The immunoreactivity was not observed in the

cardioregulatory nerves and in the heart (data not shown), but it was observed that serotonin-like immunoreactive neuronal processes ran out of the SEG and extended on the ventral skeletal muscles.

## Discussion

### Central locations of cardioresgulatory neurons

The cell bodies of cardioresgulatory neurons have not yet been identified in the CNS of any decapod species, although it has been shown that a pair of the CI nerves and two pairs of the CA nerves emerge from the thoracic ganglion in *Panulirus argus* (Maynard, 1953), *Sesarma dehaani* (Ikeya et al., 1984) and *Aniculus aniculus* (Miyazaki et al., 1985). In isopods, the neuronal cell bodies of a pair of CI axons, and of two pairs of CA (CA1 and CA2) axons, have been shown to be located in the 1st, 2nd and 3rd thoracic ganglia of *Bathynomus doederleini* (Tanaka and Kuwasawa, 1991a,b), and *Ligia exotica* (Sakurai and Yamagishi, 1998; Sakurai et al., 1999). In *S. oratoria*, we refer to the 1st ganglionic mass of the ventral nerve cord as the SEG, which consists of six segments. The cell bodies of the CI neurons are located in the 1st SEG segment. They extend their axons out of the SEG through the 10th nerve root contralateral to the cell bodies (Fig. 5), so that the axons cross over the midline. The cell bodies of both the CA1 and CA2 neurons are located in the 3rd and 4th SEG segments, respectively. The neurons extend their axons out of the SEG through the 16th and 19th nerve roots, respectively, ipsilateral to their cell bodies. These anatomical arrangements

for cardioregulatory neurons in *S. oratoria* are similar to those suggested previously in the isopods mentioned above. In both the isopods and stomatopods, the cardioregulatory cell bodies of the inhibitory, 1st and 2nd acceleratory neurons are located in successive separate central ganglia. The cell bodies of the

CI neuron are located contralaterally to their axons, and the cell bodies of the CA1 and CA2 neurons are located ipsilaterally to their axons. If the posterior part of the 1st thoracic ganglion were to be regarded as the 2nd thoracic ganglion in the isopod, *B. doederleini*, then the ganglia in which the cell bodies of the CI, CA1 and CA2 neurons are located could be expressed as the 1st, 3rd and 4th thoracic ganglia, respectively (cf. Tanaka and Kuwasawa, 1991a,b), as they are in the present animal. On the other hand, the subesophageal ganglion of stomatopods consists of the ganglia that extend nerves not only to the mouth-parts, but also to the first five pairs of thoracopods (McLaughlin, 1983). Therefore, the portion anterior to the 1st thoracic ganglion in the present material might be regarded as the subesophageal ganglion, and the 1st, 3rd and 4th segments of subesophageal ganglion in *S. oratoria* as the thoracic ganglia. It is likely that the central neuronal arrangements for cardioregulatory neurons are conserved between the taxa.

#### A common neurotransmitter of CI neurons

In the present material, GABA and histamine produced inhibitory effects on the heartbeat (Fig. 6). Picrotoxin blocked neurally induced cardiac inhibition induced by impulses of the CI nerve (Fig. 6C). Though picrotoxin is not a GABA-specific antagonist but a chloride channel blocker, the CI nerves showed immunoreactivity to anti-GABA antibodies (Fig. 10) not to anti-histamine antibodies (data not shown). These results show that GABA may be a neurotransmitter for the CI neurons in *S. oratoria*. Neurotransmitters for cardioregulatory neurons and cardiac ganglion neurons have been proposed for some crustaceans. It has been proposed that GABA may be a neurotransmitter of the CI neuron in the isopod, *B. doederleini* (Tanaka et al., 1992; F.-Tsukamoto and Kuwasawa, 2003), and in the decapods, *A. aniculus* (Yazawa and Kuwasawa, 1994) and *Panulirus argus* (Delgado et al., 2000). It is concluded that GABA may be commonly a cardio-inhibitory neurotransmitter in all those crustacean taxa.

#### Neurotransmitters for CA neurons

The heart of *S. mantis* is activated by application of E, NE (Alexandrowicz and Carlisle, 1953) and ACh (Florey and Rathmayer, 1990). While NA and DA are reported to produce

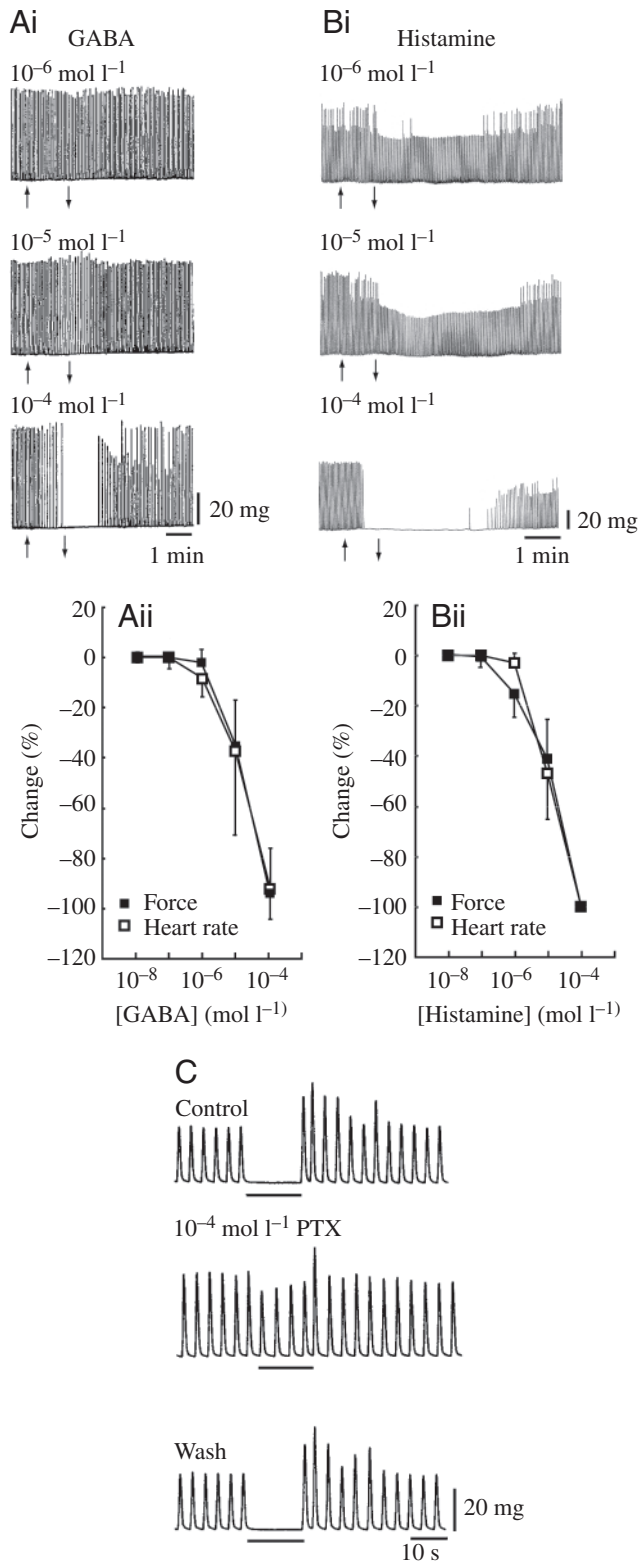


Fig. 6. (A,B) The effects of GABA (Ai) and histamine (Bi) on heartbeat of the isolated heart. (Aii, Bii) Dose-response curves for the effects on the heart rate and contraction force of the agents are plotted (values are means  $\pm$  s.d.,  $N=5$ ). GABA and histamine were applied between the arrows. Heart rate just before GABA or histamine treatment was  $35.3 \pm 12.74$  or  $31.9 \pm 13.9$ , respectively. (C) The effects of picrotoxin on cardiac inhibition induced by stimulation of the CI nerve at 70 Hz in normal saline (control; top trace), 8 min after application of  $10^{-4}$  mol l $^{-1}$  picrotoxin (middle trace) and 30 min after washing out picrotoxin (wash; bottom trace). The bars show a period of repetitive stimulation of the CI nerve. The effects of picrotoxin in abolishing neurally induced cardiac inhibition and the recovery from the effects were observed in five preparations.

cardio inhibitory activity on the heart of an isopod, *Bathynomus doederleini*, 5-HT, OA and Glu are reported to produce cardio-acceleratory activity (Tanaka et al., 1992; Yazawa et al., 1998; F.-Tsukamoto and Kuwasawa, 2003).

ACh has been proposed to be a neurotransmitter for cardio-accelerator nerves in *B. doederleini* (Tanaka et al., 1992), and DA to be a neurotransmitter for cardio-accelerator nerves in the decapod species, *A. aniculus* (Yazawa and Kuwasawa,

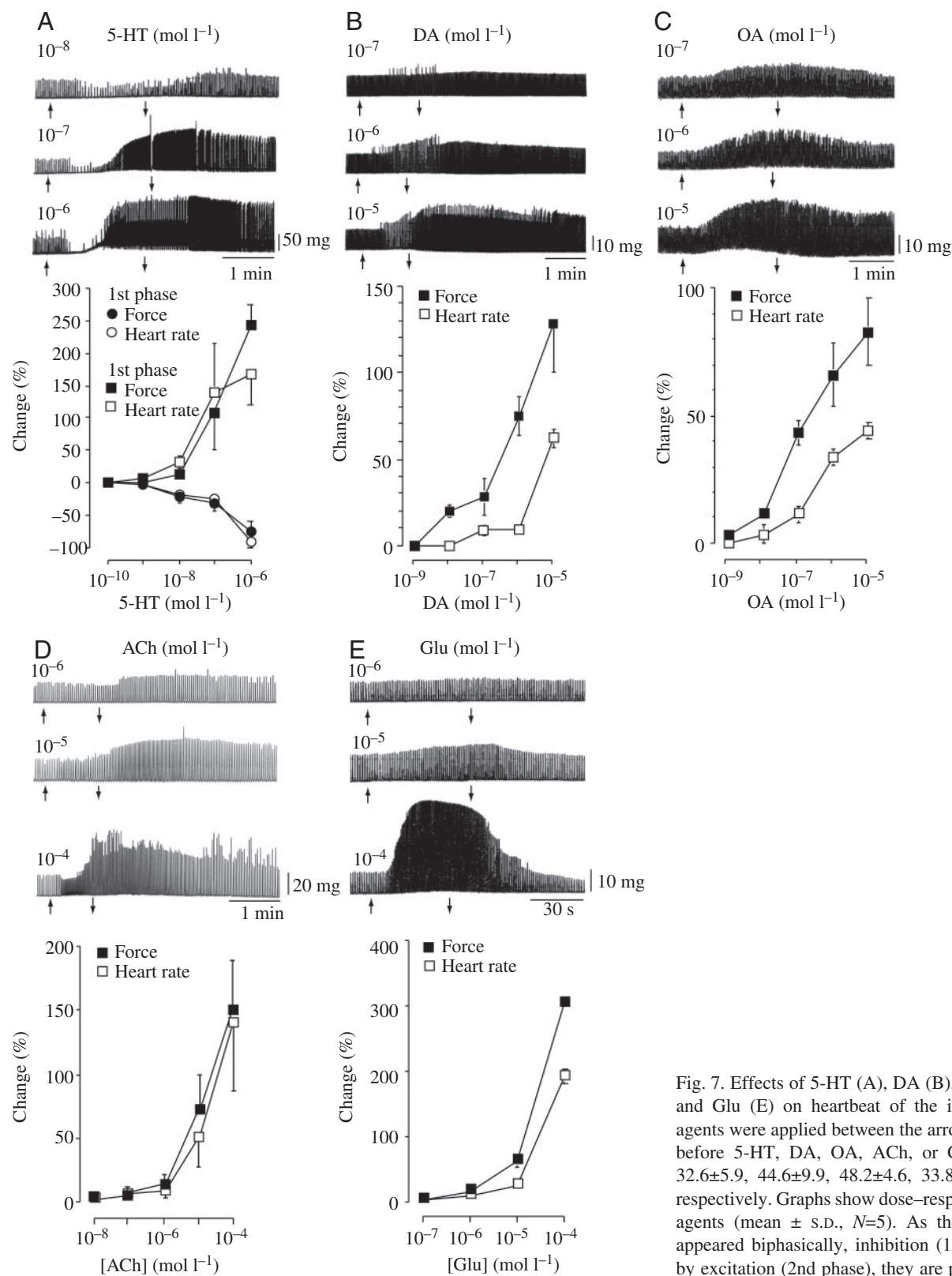


Fig. 7. Effects of 5-HT (A), DA (B), OA (C), ACh (D) and Glu (E) on heartbeat of the isolated heart. The agents were applied between the arrows. Heart rate just before 5-HT, DA, OA, ACh, or Glu treatment was  $32.6 \pm 5.9$ ,  $44.6 \pm 9.9$ ,  $48.2 \pm 4.6$ ,  $33.8 \pm 3.9$  or  $36.0 \pm 4.9$ , respectively. Graphs show dose-response curves for the agents (mean  $\pm$  s.d.,  $N=5$ ). As the effects of 5-HT appeared biphasically, inhibition (1st phase) followed by excitation (2nd phase), they are plotted separately.

1994) and the crab (Fort and Miller, 2001; reviewed by Cooke, 2002). In the present study the heart was activated by 5-HT, DA, OA and Glu, as well as E, NE and ACh (Fig. 7). No serotonin-like immunoreactivity was observed in CA axons

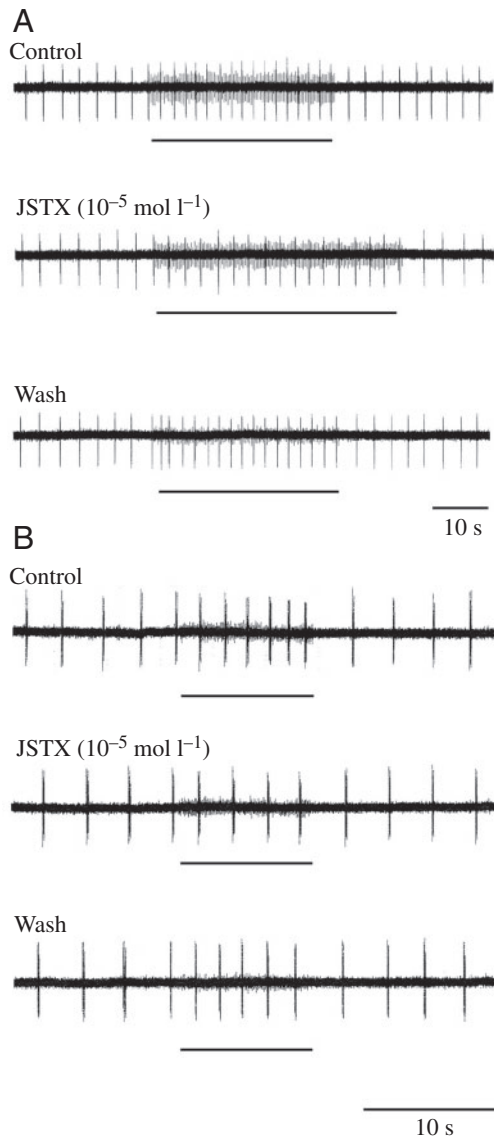


Fig. 8. (A) Effects of JSTX on cardiac acceleration induced by stimulation of the CA1 nerve at 3 Hz. Vertical lines show extracellularly recorded bursting impulses of the cardiac ganglion corresponding to heartbeats. The stimulation produced cardiac acceleration in normal saline (control; top trace). Small fine potentials during the bars in the records are stimulus artifacts. Application of JSTX ( $10^{-5}$  mol  $l^{-1}$ ) blocked the CA1-induced acceleration at 15 min after the onset of the application (middle trace). The stimulation of the CA1 nerve at 3 h after washing out JSTX induced cardiac acceleration (wash; bottom trace). The bar shows a period of repetitive stimulation. (B) Effects of JSTX on cardiac acceleration induced by stimulation of the CA2 nerve at 10 Hz. Application of JSTX blocked the CA2 induced acceleration at 30 min after the onset of the application. The stimulation of the CA2 nerve at 1 h after washing out JSTX induced cardiac acceleration. Small fine potentials during the bars in the records are stimulus artifacts.

and cardiac ganglion neurons (data not shown), although 5-HT activated heartbeat (Fig. 7A). Cardiac acceleration induced by stimulation of the CA nerves was not antagonized by cholinergic blockers, atropine and d-tubocurarine, or by catecholaminergic blockers, chlorpromazine and phentolamine (data not shown). Thus, ACh, 5-HT and catecholamines may be excluded from the list of transmitter candidates for the CA neurons.

Glu increased not only contraction force but also heart rate (Fig. 7E). JSTX blocked cardiac acceleration induced by the CA1 and CA2 nerves (Fig. 8). Glutamate-like immunoreactivity was observed in both the CA1 and CA2 axons (Fig. 11). JSTX is known to antagonize glutamatergic actions on the skeletal muscle of the lobster *Palinurus japonicus* (Kawai et al., 1982), on the stomach muscle of *S. oratoria* (Chiba and Tazaki, 1992), the heart of *L. exotica* (Sakurai et al., 1998) and on the arterial valve in *B. doederleini* (F.-Tsukamoto and Kuwasawa, 2003). These results may indicate that, in *S. oratoria*, both the CA1 and CA2 neurons are glutamatergic. It has been suggested that DA may be a neurotransmitter of the CA nerves in the hermit crab, *A. aniculus* (Yazawa and Kuwasawa, 1994) and the crab (Fort and Miller, 2001; reviewed by Cooke, 2002), while Glu had little effect on the cardiac ganglion (Yazawa and Kuwasawa, 1992, 1994). In the isopod, *B. doederleini*, ACh may be a neurotransmitter of the CA neurons (Tanaka et al., 1992).

Thus, it seems that the transmitters of cardio-accelerator nerves may be varied among the crustacean taxa.

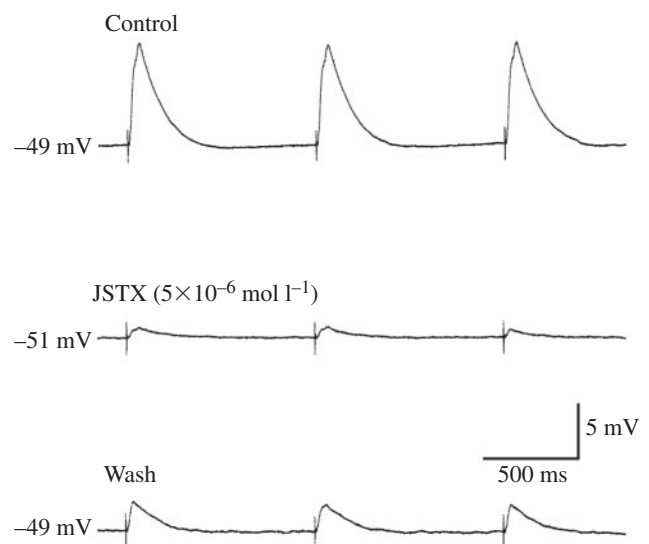


Fig. 9. Effects of JSTX ( $5 \times 10^{-6}$  mol  $l^{-1}$ ) on EJP in myocardial cells. The EJPs were evoked by stimuli applied to the cardiac ganglion trunk at 1 Hz. The EJPs in normal saline (control) were blocked at 30 min after the onset of JSTX application ( $5 \times 10^{-6}$  mol  $l^{-1}$  JSTX). The EJPs partially recovered at 120 min after washing out of JSTX (wash). The numbers on the left side of the traces show the membrane potentials of the cardiac muscle at the beginning of the recordings.

### Neurotransmitters for cardiac ganglion neurons

EJPs in the myocardium induced by motor impulses of the cardiac ganglion were blocked by JSTX (Fig. 9). Glutamate-like immunoreactivity was observed in all of the 15 cardiac ganglion neurons (Fig. 11). On the other hand, heartbeat triggered by the cardiac ganglion neurons was not antagonized by the cholinergic blockers and catecholaminergic blockers (data not shown). These results may indicate that, in *S. oratoria*, the cardiac ganglion neurons are glutamatergic. Pharmacological experiments suggested that ACh and DA may be neurotransmitters of small (pacemaker) and large (motor) neurons of the cardiac ganglion, respectively, although Glu exerted little effect on the cardiac ganglion and cardiac muscle in the hermit crab *A. aniculus* (Yazawa and Kuwasawa, 1992, 1994). On the other hand, glutamate-like immunoreactivity has been observed in the small and large neurons of the cardiac ganglion in the lobster *P. argus* (Delgado et al., 2000). A neurotransmitter of the cardiac ganglion neurons appears to differ in the anomuran (*A. aniculus*) and palinuran (*P. argus*) decapod species examined. Since Glu increased contraction force but not heart rate in the isopod, *B. doederleini*, Glu was proposed to be a neurotransmitter of the cardiac ganglion neurons (Yazawa et al., 1998). Glutamate-like immunoreactivity has been observed in the motor neurons of the cardiac ganglion in the isopod, *L. exotica* (Sakurai et al., 1998). Glutamate may be a neurotransmitter of the cardiac ganglion neurons of *S.*

*oratoria* as of the case in a decapod, *P. argus*, and in isopods, *B. doederleini* and *L. exotica*.

### Neurohormones for control of the heart

#### Cardiac excitation

Alexandrowicz (1952) observed that, in *S. mantis*, unpaired nerves ran in the connectives between the subesophageal, thoracic and abdominal ganglia, and extended toward the ventral body muscles. He called this nervous system 'the system of the median connectives' and suggested that it had a neurosecretory function. He (Alexandrowicz, 1953) also observed neuropile-like networks on the inner surface of the pericardium in *S. mantis* and referred to it as the pericardial organ. The pericardial organ contained neuronal cell bodies and endings of nerves extending from the CNS. Pericardial organ extracts increased heart rate and contraction force in *S. mantis* (Alexandrowicz and Carlisle, 1953; Brown, 1964b). Substances contained in the pericardial organ and in the system of the median connectives have not yet been examined in any stomatopod species. The decapod pericardial organ contains 5-HT, DA, OA and several neuropeptides (Cooke and Sullivan, 1982; Cooke, 1988; Christie et al., 1995; reviewed by Cooke, 2002). In the present species, 5-HT, DA and OA increased heart rate and contraction force (Fig. 7A–C). Since we observed serotonin-like immunoreactivity in the system of the median connectives as well as the CNS (data not shown), 5-HT may be one of the cardioexcitatory neurohormones. ACh may be a

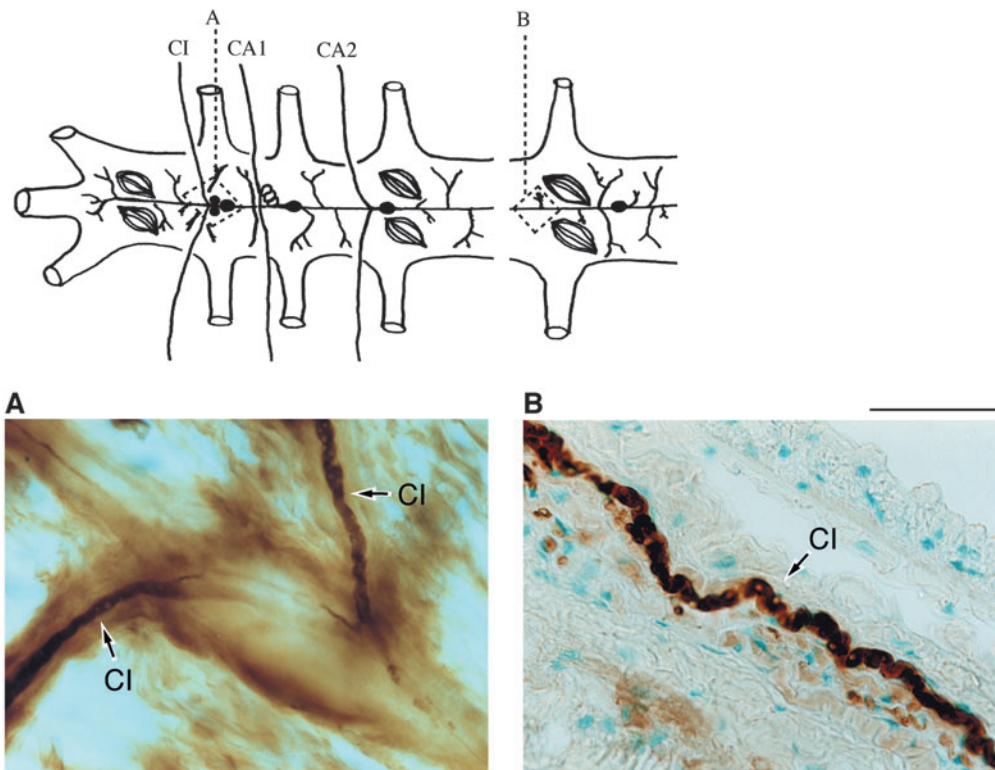


Fig. 10. Photographs of CI axons in whole-mount preparations and paraffin sections of the heart. (A) A pair of bilateral CI axons, which show GABA-like immunoreactivity, join the cardiac ganglion in a whole-mount preparation. (B) A CI axon running in the trunk of the cardiac ganglion showed GABA-like immunoreactivity in a paraffin section. Scale bar, 50  $\mu$ m. The diagram shows the areas depicted in A and B.

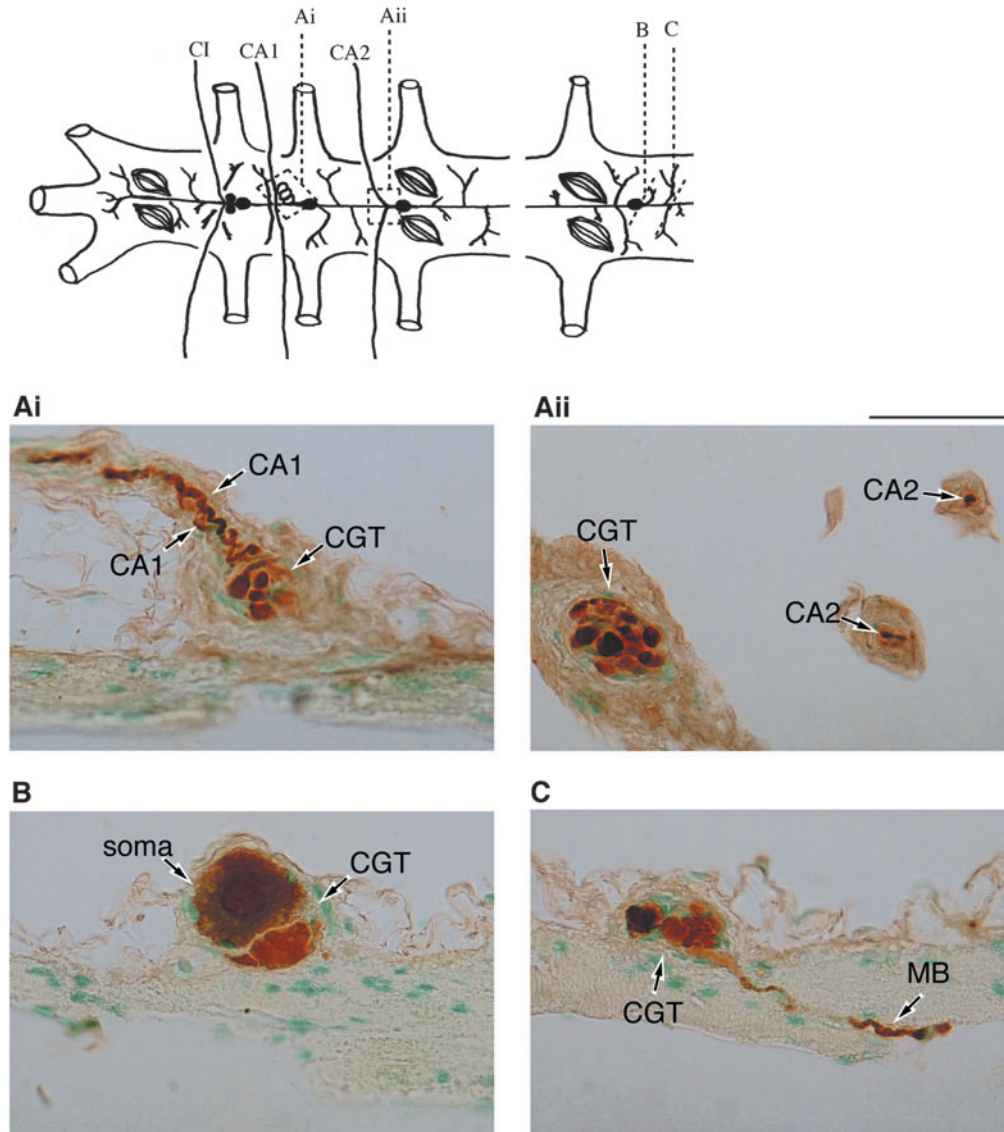


Fig. 11. Photographs of glutamate-like immunoreactivity in paraffin sections obtained from the heart. Glutamate-like immunoreactivity is seen in (Ai) the CA1 nerve and the cardiac ganglion trunk (CGT) and (Aii) the CA2 nerves on both sides and the CGT. (B) Glutamate-like immunoreactivity in the soma of the cardiac ganglion neuron. (C) Glutamate-like immunoreactivity in a major branch (MB) of the cardiac ganglion extending to the myocardium. Scale bar, 50  $\mu$ m. The diagram shows the areas depicted in A–C.

neurotransmitter for small neurons of the pacemaker in the cardiac ganglion of the hermit crabs (Yazawa and Kuwasawa, 1992) and for the CA axons of the isopod (Tanaka et al., 1992). It is suggested that in the stomatopod a transmitter of CA neurons is Glu but not ACh, although ACh increased both heart rate and contraction force (Fig. 7D). It is of interest for further study to determine whether ACh is contained as a neurohormonal substance in the pericardial organ of *S. oratoria*.

#### Cardiac inhibition

Cardio-inhibitory substances other than GABA have not previously been reported in any stomatopod species. This study showed that HA induced inhibitory effects on the heart (Fig. 6B). However, no histamine-like immunoreactivity was

observed in the CI neurons. Since many histamine-like immunoreactive neurons were located throughout the CNS in the present material (data not shown), it cannot be excluded that HA is possibly a cardio-inhibitory neurohormone, as proposed for *Homarus americanus* (Hashemzadeh-Gargari and Freschi, 1992).

#### List of abbreviations

AA	anterior artery
ACh	acetylcholine
AG1–6	1st to 6th abdominal ganglia
CA1 and CA2	1st and 2nd cardio-accelerator
Cer. G	cerebral ganglion

Ceph.-Thorax	cephalo-thorax
CG	cardiac ganglion
CGC	cardiac ganglion cell
CGT	cardiac ganglion trunk
CI	cardio-inhibitor
CNS	central nervous system
DA	dopamine
DAB	3,3'-diaminobenzidine-tetrahydrochloride
DW	distilled water
E	epinephrine
ECG	electrocardiogram
EJP	excitatory junctional potential
GABA	$\gamma$ -amino-butyric acid
Glu	glutamate
HA	histamine
5-HT	serotonin
JSTX	joro-spider toxin
LA1-15	1st to 15th lateral arteries
MB	major branch
NB	neural branch
NE	norepinephrine
NR	nerve root
PA	posterior artery
PAP	peroxidase-antiperoxidase
PBT	phosphate buffer containing Triton X-100
PTX	picrotoxin
SEG	subesophageal ganglion
SW	seawater
TG7-9	7th to 9th thoracic ganglia

This study was partly supported by the grant from JSPS No. 12304050 (K.K.). We thank Mr K. Shishikura for generously collecting animals. We would like to thank Prof. R. B. Hill, University of Rhode Island, for revision of this manuscript. Contribution from the Shimoda Marine Research Center, No. 707.

## References

- Alexandrowicz, J. S. (1932). The innervation of the heart of Crustacea. I. Decapoda. *Q. J. Microsc. Sci.* **75**, 181-249.
- Alexandrowicz, J. S. (1934). The innervation of the heart of Crustacea. II. Stomatopoda. *Q. J. Microsc. Sci.* **76**, 511-548.
- Alexandrowicz, J. S. (1952). Notes on the nervous system in the Stomatopoda. I. The system of median connectives. *Publ. Stn. Zool. Napoli* **23**, 201-214.
- Alexandrowicz, J. S. (1953). Notes on the nervous system in the Stomatopoda. II. The system of dorsal trunks. III. Small nerve cells in motor nerves. *Publ. Stn. Zool. Napoli* **24**, 29-45.
- Alexandrowicz, J. S. and Carlisle, D. B. (1953). Some experiments on the function of the pericardial organs in Crustacea. *J. Mar. Biol. Assn. UK* **32**, 175-192.
- Ando, H. and Kuwasawa, K. (1993). Central origins and functions of extrinsic cardiac nerves in the stomatopod crustacean *Squilla oratoria*. *Zool. Sci.* **10**, Suppl., 94.
- Ando, H. and Kuwasawa, K. (1994). Identification of cardioinhibitory and cardioacceleratory neurons in the CNS of the stomatopod crustacean *Squilla oratoria*. *Zool. Sci.* **11**, Suppl., 95.
- Ando, H., Kuwasawa, K., Yazawa, T. and Kurokawa, M. (1995). An analysis of cardio-regulatory nerves in the stomatopod crustacean *Squilla oratoria*. *Physiol. Zool.* **68**, 75.
- Brown, H. F. (1964a). Electrophysiological investigations of the heart of *Squilla mantis*. I. The ganglionic nerve trunk. *J. Exp. Biol.* **41**, 689-700.
- Brown, H. F. (1964b). Electrophysiological investigations of the heart of *Squilla mantis*. III. The mode of action of pericardial organ extract on the heart. *J. Exp. Biol.* **41**, 723-734.
- Chiba, C. and Tazaki, K. (1992). Glutamatergic motoneurons in the stomatogastric ganglion of the mantis shrimp *Squilla oratoria*. *J. Comp. Physiol. A* **170**, 773-786.
- Christie, A. E., Skiebe, P. and Marder, E. (1995). Matrix of neuromodulators in neurosecretory structures of the crab *Cancer borealis*. *J. Exp. Biol.* **198**, 2431-2439.
- Cooke, I. M. (1988). Studies on the crustacean cardiac ganglion. *Comp. Biochem. Physiol.* **91C**, 205-218.
- Cooke, I. M. (2002). Reliable, responsive pacemaking and pattern generation with minimal cell numbers: the crustacean cardiac ganglion. *Biol. Bull.* **202**, 108-136.
- Cooke, I. M. and Sullivan, R. E. (1982). Hormones and Neurosecretion. In *The Biology of Crustacea*, vol. 3 (ed. H. L. Atwood and D. C. Sandeman), pp. 205-290. New York: Academic Press.
- Delgado, J. Y., Oyola, E. and Miller, M. W. (2000). Localization of GABA- and glutamate-like immunoreactivity in the cardiac ganglion of the lobster *Panulirus argus*. *J. Neurocytol.* **29**, 605-619.
- F.-Tsukamoto, Y. and Kuwasawa, K. (2003). Neurohormonal and glutamatergic neuronal control of the cardioarterial valves in the isopod crustacean *Bathynomus doederleini*. *J. Exp. Biol.* **206**, 431-443.
- Florey, E. and Rathmayer, M. (1990). Facilitation and potentiation of transmitter release at neuromuscular synapses in the heart of *Squilla mantis*; functional and theoretical implications. In *Frontiers in Crustacean Neurobiology* (ed. K. Wiese, W.-D. Krenz, J. Tautz, H. Reichert and B. Mulloney), pp. 330-337. Basel: Birkhäuser Verlag.
- Fort, T. J. and Miller, M. W. (2001). Functional organization of the cardiac system of the blue crab *Callinectes Sapidus*: GABAergic and catecholaminergic regulatory fibers. Abstract, 2001 Society for Neuroscience.
- Hashemzadeh-Gargari, H. and Freschi, J. E. (1992). Histamine activates chloride conductance in motor neurons of the lobster cardiac ganglion. *J. Neurophysiol.* **68**, 9-15.
- Ikeya, H., Uesaka, H., Yamagishi, H. and Ebara, A. (1984). Studies on the cardioregulatory nerves in a crab, *Sesarma dehaani*. *Comp. Biochem. Physiol.* **78A**, 743-748.
- Irisawa, H. and Irisawa, A. F. (1957). The electrocardiogram of a stomatopod. *Biol. Bull.* **112**, 358-362.
- Kawai, N., Niwa, A. and Abe, T. (1982). Spider venom contains specific receptor blocker of glutaminergic synapses. *Brain Res.* **247**, 169-171.
- Kihara, A. and Kuwasawa, K. (1984). A neuroanatomical and electrophysiological analysis of nervous regulation in the heart of an isopod crustacean, *Bathynomus doederleini*. *J. Comp. Physiol. A* **154**, 883-894.
- Maynard, D. M., Jr (1953). Activity in a crustacean ganglion. I. cardio-inhibition and acceleration in *Panulirus argus*. *Biol. Bull.* **104**, 156-170.
- Maynard, D. M. (1960). Circulation and heart function. In *The Physiology of Crustacea*, vol. 1 (ed. T. H. Waterman), pp.161-226, New York: Academic Press.
- McLaughlin, P. A. (1980). Order Stomatopoda. In *Comparative Morphology of Recent Crustacea*, pp. 63-69. San Francisco: Freeman.
- McLaughlin, P. A. (1983). Internal anatomy. In *The Biology of Crustacea*, vol. 5 (ed. L. H. Mantel), pp. 1-52. New York: Academic Press.
- Miyazaki, T., Kuwasawa, K., Yazawa, T. and Mashimo, K. (1985). Identification of the cardio-regulator nerves in a marine hermit crab and the shadow-induced cardiac inhibition in some decapods. *Zool. Sci.* **2**, 35-47.
- Quicke, D. L. J. and Brace, R. C. (1979). Differential staining of cobalt- and nickel-filled neurones using rubeanic acid. *J. Microsc.* **115**, 161-163.
- Sakurai, A. and Yamagishi, H. (1998). Identification of two cardio-acceleratory neurons in the isopod crustacean, *Ligia exotica* and their effects on cardiac ganglion cells. *J. Comp. Physiol. A* **182**, 145-152.
- Sakurai, A., Mori, A. and Yamagishi, H. (1998). Glutamatergic neuromuscular transmission in the heart of the isopod crustacean *Ligia exotica*. *J. Exp. Biol.* **201**, 2833-2842.
- Sakurai, A., Mori, A. and Yamagishi, H. (1999). Cardioinhibitory neurons in the isopod crustacean *Ligia exotica*. *Zool. Sci.* **16**, 401-405.
- Tanaka, K. and Kuwasawa, K. (1991a). Identification of cardio-acceleratory neurons in the thoracic ganglion of the isopod crustacean *Bathynomus doederleini*. *Brain Res.* **544**, 311-314.
- Tanaka, K. and Kuwasawa, K. (1991b). Identification of cardio-inhibitory

- neurons in the thoracic ganglion of the isopod crustacean *Bathynomus doederleini*. *Brain Res.* **558**, 339-342.
- Tanaka, K., Yazawa, T. and Kuwasawa, K.** (1992). Cholinergic and GABAergic control of the heart in the isopod crustacean, *Bathynomus doederleini*. In *Phylogenetic Models in Functional Coupling of the CNS and the Cardiovascular System, Comparative Physiology*, vol. 11 (ed. R. B. Hill, K. Kuwasawa, R. R. McMahon and T. Kuramoto), pp. 132-140. Basel: Karger.
- Watanabe, A., Obara, S. and Akiyama, T.** (1967). Pacemaker potentials for the periodic burst discharge in the heart ganglion of a stomatopod, *Squilla oratoria*. *J. Gen. Physiol.* **50**, 839-862.
- Watanabe, A., Obara, S. and Akiyama, T.** (1968). Inhibitory synapses on pacemaker neurons in the heart ganglion of a stomatopod, *Squilla oratoria*. *J. Gen. Physiol.* **52**, 908-924.
- Watanabe, A., Obara, S. and Akiyama, T.** (1969). Acceleratory synapses on pacemaker neurons in the heart ganglion of a stomatopod, *Squilla oratoria*. *J. Gen. Physiol.* **54**, 212-231.
- Yazawa, T. and Kuwasawa, K.** (1984). The cardio-regulator nerves of the hermit crabs: anatomical and electrophysiological identification of their distribution inside the heart. *J. Comp. Physiol. A* **154**, 871-881.
- Yazawa, T. and Kuwasawa, K.** (1992). Intrinsic and extrinsic neural and neurohumoral control of the decapod heart. *Experientia* **48**, 834-840.
- Yazawa, T. and Kuwasawa, K.** (1994). Dopaminergic acceleration and GABAergic inhibition in extrinsic neural control of the hermit crab heart. *J. Comp. Physiol. A* **174**, 65-75.
- Yazawa, T., Tanaka, K., Yasumatsu, M., Otokawa, M., Aihara, Y., Ohsuga, K. and Kuwasawa, K.** (1998). A pharmacological and HPLC analysis of the excitatory transmitter of the cardiac ganglion in the heart of the isopod crustacean *Bathynomus doederleini*. *Can. J. Physiol. Pharmacol.* **76**, 599-604.