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Neurohormonal and glutamatergic neuronal control of the cardioarterial valves in the isopod crustacean *Bathynomus doederleini*

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Summary

The heart of *Bathynomus doederleini* gives rise to an anterior median artery (AMA), one pair of anterior lateral arteries (ALAs) and five pairs of lateral arteries (LAs). Cardioarterial valves are located at the junctions between the heart and arteries, each composed of a pair of muscular flaps. All valves of the AMA and the ALAs receive valve excitatory (constrictor) nerves (VEs). The valves of the ALAs receive dual innervation from both constrictor and inhibitor (dilator) nerves, while the valves of the AMA receive innervation from a constrictor nerve alone. The effects of candidate neurohormones on cardioarterial valves were examined by measuring the pressure in each artery at which haemolymph flows out of the heart through the valve. Serotonin, octopamine, norepinephrine, glutamate (Glu) and proctolin constricted the cardioarterial valves and thus decreased the arterial pressure in all the arteries. Dopamine also decreased the arterial pressure of arteries except for the ALAs, in which pressure was increased. Among the neurohormones exerting excitatory effects on the valves, only Glu depolarized the membrane potential of valve muscle cells. The glutamatergic agonists kainate and quisqualate also depolarized the valve muscle cells of the AMA. Excitatory junctional potentials produced in the valves of the AMA in response to the stimulation of a VE were blocked by the glutamatergic antagonists Joro spider toxin and MK-801. Glu is the likeliest candidate for a neurotransmitter for the VEs.

Key words: neuronal control, neurotransmitter, cardioarterial valves, neurohormone, serotonin, dopamine, glutamate, glutamatergic agonist, glutamatergic antagonist, Joro spider toxin, valve excitatory nerve, isopod crustacean, *Bathynomus doederleini*.

Introduction

It is well known that blood flow in mammals is modulated by control of the state of vascular smooth muscle through neural control and by various vasoconstrictor and vasodilator substances. Among invertebrates, insects (Sanger and McCann, 1968) and annelids (Gardiner, 1992; Jamieson, 1992) are known to have muscle fibres in arterial walls. In the sea hare *Aplysia californica*, vasoconstrictor and vasodilator neurons innervating the arteries have been identified and are known to control haemolymph distribution to vascular beds (reviewed by Koester and Koch, 1987; Skelton et al., 1992; Brownell and Ligman, 1992). By contrast, there is no muscle in the crustacean arterial wall (reviewed by Maynard, 1960) except for the dorsal abdominal artery in decapods (Burnett, 1984; Martin et al., 1989; Wilkens et al., 1997). Haemolymph flow in arteries of crustaceans is regulated by valves at the origins of arteries or arterioles.

Alexandrowicz (1932, 1934, 1952) reported that there are innervated cardioarterial valves, located at the junctions between the heart and the arteries, in decapod, stomatopod and isopod Crustacea. Alexandrowicz (1932) concluded from anatomical studies that the valve nerves might function as valve constrictors to prevent backward flow of haemolymph during diastolic periods. The first physiological studies of crustacean cardioarterial valves were carried out by Kihara and Kuwasawa (1984) and Kihara et al. (1985). They used the isopod species *Bathynomus doederleini*, the same species investigated in the present study. One anterior median artery (AMA), one pair of anterior lateral arteries (ALAs) and five pairs of lateral arteries (LAs) arise from the heart of *Bathynomus* (Fig. 1). The cardioarterial valves each consist of a pair of muscular flaps, located at the junction between the heart and the arteries. When the valve flaps contract or relax, the aperture becomes smaller or larger, respectively, thus regulating cardiac outflow through the valves into individual arteries.

The anterior cardiac nerve (ACN) is a combined nerve comprising five nerves [valve excitatory nerve (VE), valve inhibitory nerve (VI), cardioinhibitory nerve (CI), and the first and second cardioacceleratory nerves (CA1 and CA2); Fig. 1]. As all the nerves are single axon nerves, the ACN (cf. Figs 9, 11) is composed of five axons; the valve excitatory axon, valve inhibitory axon, cardioinhibitory axon, and the first and second cardioacceleratory axons.

The valve of the AMA receives a pair of excitatory nerves

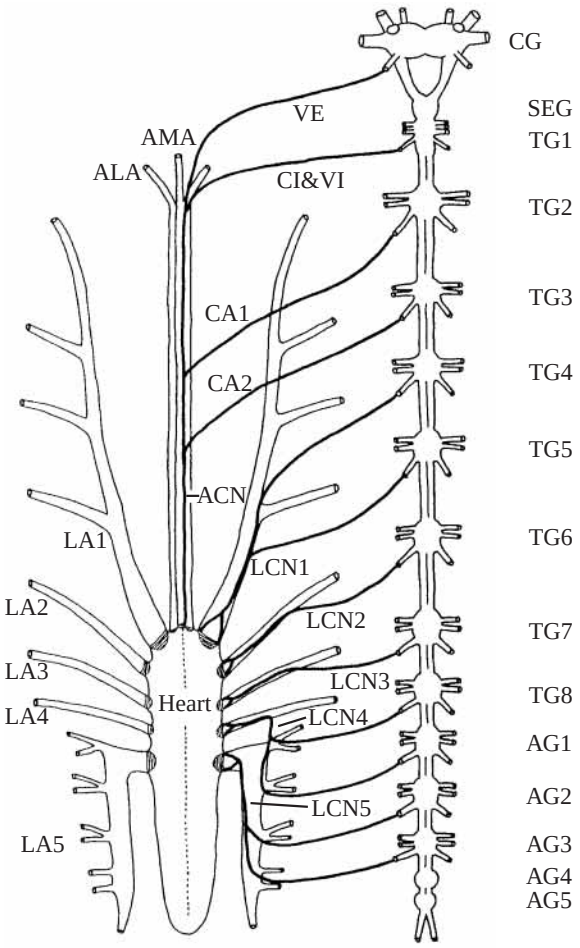


Fig. 1. Schematic drawing of the cardiovascular system and central nervous system (CNS). The right and left sides of the CNS are reversed for the sake of illustrating convenience. Cardiac nerves are shown by bold lines. The cardioarterial valves are located at the junctions between the heart and each artery. ACN, anterior cardiac nerve; AG1–5, first–fifth abdominal ganglion; ALA, anterior lateral artery; AMA, anterior median artery; CA, cardioacceleratory nerve; CI&VI, combined nerve of cardioinhibitory nerve and valve inhibitory nerve; CG, cerebral ganglion; VE, valve excitatory nerve; LA1–5, first–fifth lateral artery; LCN1–5, first–fifth lateral cardiac nerve; SEG, suboesophageal ganglion; TG1–8, first–eighth thoracic ganglion. Modified from Tanaka et al. (1996).

alone. The valve of the ALA receives dual innervation from two pairs of both excitatory and inhibitory nerves, while the valves of all the five lateral arteries receive inhibitory nerves alone (Kihara et al., 1985; Fujiwara-Tsukamoto et al., 1992). The valve excitatory nerves produce excitatory junctional potentials (EJPs) in valve muscles, causing contraction of a valve, and a resulting decrease of haemolymph outflow into the artery. The valve inhibitory nerves produce cholinergic inhibitory junctional potentials (IJPs) in valve muscles, causing relaxation of the valve, and a resulting increase of haemolymph flow through the valve (Okada et al., 1997). A candidate neurotransmitter for the valve excitatory nerve has not yet been determined. This requires pharmacological experiments, combined with electrophysiological recording of the membrane potential and EJPs.

Glutamate (Glu), γ-aminobutyric acid (GABA) and acetylcholine (ACh) are established neurotransmitters in crustaceans (reviewed by Atwood, 1982). ACh is a proposed neurotransmitter for valve inhibitory nerves of *Bathynomus* (Okada et al., 1997). Glu has been shown to be a neurotransmitter for cardiac ganglionic cells in the isopods *Bathynomus* (Yazawa et al., 1998) and *Ligia* (Sakurai et al., 1998) and in several decapod species (Benson, 1981; Delgado et al., 2000; reviewed by Cooke, 2002). GABA is a proposed neurotransmitter of cardioinhibitory nerves in *Bathynomus* (Tanaka et al., 1992). In decapods, GABA is also a proposed neurotransmitter for the inhibitory nerves innervating the valves of the lateral abdominal arteries (*Homarus americanus*; Davidson et al., 1998). We examined the pharmacological effects of these neurotransmitters on valve muscle of the AMA, ALA and the fifth LA (LA5; as a representative of all lateral arteries).

The valve of LA5 does not receive excitatory innervation. However, pressure pulses in LA5 are decreased by application of serotonin [5-hydroxytryptamine (5-HT)] or octopamine (OA). For this reason, Fujiwara and Kuwasawa (1987) postulated that there is hormonal regulation of haemolymph flow *via* the cardioarterial valve. 5-HT, OA and dopamine (DA) are known as neurohormones stored in the central nervous system and in the pericardial organ of the decapod pericardial cavity (reviewed by Cooke and Sullivan, 1982; Cooke, 2002). Peptides, such as proctolin (Proc), are stored in the pericardial organ of the crab *Cancer borealis* (Christie et al., 1995). The pericardial organ releases these monoamines and peptides to regulate heart activity (reviewed by Cooke and Sullivan, 1982). 5-HT, OA and Proc, which have excitatory effects on the heartbeat, also act on the cardioarterial valves of the lobster *Panulirus japonicus* (Kuramoto and Ebara, 1984). Norepinephrine (NE) has been detected in the cardiac ganglion of *H. americanus* (Ocorr and Berlind, 1983). Therefore, we included these putative neurohormones (5-HT, OA, DA, NE and Proc) of decapods in the present study in order to examine neurohormones that might act on the cardioarterial valve of *Bathynomus*.

Bathynomus haemolymph is delivered to specific arteries under neural control in a behaviour-adaptive manner. For example, during swimming, impulse rates of the fifth lateral cardiac nerve (LCN5) and of the valve excitatory axon increase, while the impulse rate of the first lateral cardiac nerve (LCN1) decreases (Tanaka et al., 1996; Fujiwara-Tsukamoto et al., 1992; Okada and Kuwasawa, 1995). These changes in impulse rate of the valve nerves cause an increase of haemolymph flow into LA5, which supplies haemolymph to the swimmeret muscle. In decapods, evidence for neural regulation of haemolymph distribution has not been demonstrated, but it is reported that injection of neurohormones into the body exerts a variety of patterns of changes in haemolymph flow among arteries of *Cancer*

magister (Airriess and McMahon, 1992; McGow et al., 1994; McGow and McMahon, 1995). It is well known that 5-HT and OA are relevant to specific behavioural states in the lobster *H. americanus* (Livingstone et al., 1980).

The present study was designed to test the responses to crustacean neurohormonal substances of all the cardioarterial valves of *Bathynomus*. We demonstrate here that crustacean hormones have strong effects on the cardioarterial valves of *Bathynomus*. Evidence for hormonal regulation of haemolymph distribution in *Bathynomus* will contribute to understanding regulation of circulation at the level of organismal behaviour. Our data also identify a candidate neurotransmitter for the valve excitatory nerve, which has constrictor effects on the valves of the anterior arteries (AMA and right and left ALAs). Some of these results have appeared in abstract form (F.-Tsukamoto and Kuwasawa, 1995; F.-Tsukamoto and Kuwasawa, 1997).

Materials and methods

Living specimens of *Bathynomus doederleini* Ortmann were collected in fishing traps at Sagami Bay and off Tokyo Bay, Japan. They were then held in tanks of recirculated seawater, kept at 15°C, in the laboratory's aquarium until used. More than 100 specimens, measuring 9–15 cm in length, were used in this study.

Arterial pressure

Before dissection, animals were anaesthetised with an isotonic (0.36 mol l^{-1}) $MgCl_2$ solution injected into the body. The cephalon, thoracic appendages, sternum, digestive organs and reproductive organs were removed carefully. The remaining specimen contained the major parts of the cardiovascular system attached to the carapaces. This was pinned ventral side up in a Sylgard (Dow Corning)-lined experimental chamber filled with filtered seawater (SW).

The pressure of the artery was monitored with a pressure transducer (P50, Gould Stratham Instruments Inc., Hato Rey, Puerto Rico) connected to a polyethylene cannula (tip diameter, 250 μm) inserted into the artery distal to the valve. Two types of preparations were made for recording the pressure of the arteries. In the first type of preparation, pressure pulses were recorded from an artery, corresponding to heartbeat in a one-to-one manner. In this preparation, all arteries, except for the artery used for recording, were ligated at the junction between the heart and arteries. This removed any influence derived from opening or closing of the valves of the other arteries. The heart was internally perfused with SW by cannulation into the heart through a small opening on the ventral side.

The second type of preparation was used for recording arterial pressure without heartbeat. The tip of the perfusion cannula was directed towards the valve from the inside of the heart and tied to pieces of heart muscle surrounding the cannula. In these preparations, heartbeat was almost lacking in the mechanograms, although some preparations showed small oscillatory contractions of ligated cardiac muscles near the valve.

Experiments were performed at room temperature (approximately 22°C).

Membrane potential of valve muscle

A preparation of nerve and valve muscle was used to record intracellular junctional potentials. The cardiovascular system, together with the carapace, was pinned in an experimental chamber, dorsal side up. The anterior cardiac nerve, which runs along the dorsal side of the anterior arteries (Fig. 1), was exposed by removing several pieces of carapace just above the junction between the heart and the anterior arteries. The preparation, containing the set of three cardioarterial valves – located at the junction between the heart and anterior arteries – and short lengths of the basal portions of the anterior arteries, was isolated together with a pair of 'semi-isolated' anterior cardiac nerves. We used a glass suction electrode to apply electrical stimuli to the anterior cardiac nerve, which was cut and partially isolated from the arterial wall.

The ventral wall of the anterior median artery was cut open longitudinally to expose the valve. These preparations were pinned to the bottom of the chamber (capacity of approximately 400 μl) and superfused at a flow rate of 1–2 ml min^{-1} with chilled *Bathynomus* saline to maintain the bath temperature at 15–20°C. The composition of the saline was: 479.4 mmol l^{-1} Na^+; 15.7 mmol l^{-1} K^+; 14.6 mmol l^{-1} Ca^{2+}; 60.7 mmol l^{-1} Mg^{2+}; 627.6 mmol l^{-1} Cl^-; 9.1 mmol l^{-1} $SO4^{2-}$; 5 mmol l^{-1} Hepes, pH 7.9 (F.-Tsukamoto et al., 2000).

Application of agents

All agents were dissolved in SW or *Bathynomus* saline. All except Joro spider toxin (JSTX) were applied by means of turning the switch of a three-way valve in the perfusion line. The bars in figures show the time between turning on and off the perfusion valve. JSTX was applied as droplets directly to preparations using a micropipette.

Chemicals

The following agents were used: acetylcholine chloride, dopamine hydrochloride, serotonin creatinine sulfate, γ–amino-n-butyric acid, sodium L(+)-glutamate monohydrate, Joro spider toxin (JSTX-3), (Wako Pure Chemical Industries, Osaka, Japan), l-norepinephrine bitartrate, octopamine hydrochloride, proctolin acetate salt, kainic acid hydrate, quisqualic acid (Sigma Chemicals, St Louis, USA), (+)-MK-801 hydrogen maleate (RBI, Natick, USA).

Electrophysiology

A glass capillary probe was placed on the ventral side of the heart for recording of cardiac mechanograms. The probe was mounted on a strain gauge mechanotransducer (TB611T, Nihon Kohden, Tokyo, Japan). Intracellular recording from valve muscle cells was carried out with a glass microelectrode filled with 3 mol l^{-1} KCl (tip resistance, 8–20 MΩ). The intracellular microelectrode, connected to an Ag–AgCl wire, was coupled to a high-input impedance preamplifier (MEZ-8300, Nihon Kohden). Signals were displayed on a pen-writing

chart recorder (WI-641G, Nihon Kohden) and on an oscilloscope (VC-11, Nihon Kohden). Signals on the oscilloscope display were photographed with an oscilloscope camera (RLG-6201, Nihon Kohden).

For stimulating valve nerves, the distal cut-stump of the nerve was introduced into a glass suction electrode connected to an Ag–AgCl wire. A stimulating electrode was connected to a pulse generator (SEN-7203, Nihon Kohden) *via* an isolator (SS-302J, Nihon Kohden).

Immunocytochemistry

The rabbit polyclonal anti-GABA antibody and the mouse monoclonal anti-Glu antibody were purchased from INCSTAR Corp. (Stillwater, USA). The instructions for the anti-Glu antibody from INCSTAR Corp. stated that the antibody had 3% crossreactivity to GABA compared with Glu.

Paraffin sections and whole-mount preparations were used for immunocytochemical studies. For paraffin sections, isolated anterior cardiac nerves with arterial wall were fixed with 4% paraformaldehyde : 0.3% glutaraldehyde in 100 mmol l^{-1} sodium phosphate buffer containing 15% sucrose (pH 7.4) for 2–5 h at 4°C. After fixation, they were rinsed with 100 mmol l^{-1} phosphate buffer containing 15% sucrose overnight at 4°C and were embedded in paraffin. 10 μm serial paraffin sections were cut, dried and deparaffinized by xylene, rehydrated through an ethanol series and immersed in distilled water (DW). In order to avoid intrinsic peroxidase activity in the sectioned tissues, the slides were treated with 0.3% H_2O_2 in DW for 30 min at room temperature, incubated with 100 mmol l^{-1} phosphate buffer containing 0.1% Triton X-100 (0.1%-PBT; pH 7.2) for 15 min. The slides were treated with the primary antibodies, rabbit polyclonal anti-GABA antibody or mouse monoclonal anti-Glu antibody, diluted 1:2000 and 1:1000, respectively, in 0.1%-PBT, for 20–24 h at 4°C and washed out with 0.1%-PBT. Specimens were treated with the secondary antibody of goat anti-rabbit immunoglobulin G (IgG; Sigma) for GABA and anti-mouse IgG (Sigma) for Glu, diluted 1:200 in 0.1%-PBT, for 2 h at room temperature and washed out with 0.1%-PBT. The third antibody, rabbit (for GABA) or mouse (for Glu) peroxidase–antiperoxidase (PAP) complex (Sigma), diluted 1:200 in 0.1%-PBT, was then applied for 2 h. The slides were rinsed with 0.1%-PBT, then 0.03% 3,3′-diaminobenzidine-tetrahydrochloride (DAB) in 50 mmol l^{-1} Tris buffer (pH 7.6) containing 0.003% H_2O_2 was applied for approximately 10 min at room temperature in the dark. The peroxidase reaction was stopped by transferring the slides to DW. Sections were counterstained with methyl green, dehydrated through an ethanol series, cleared in xylene and mounted in Bioleit (Ohken Shoji, Tokyo, Japan) on slides.

For whole-mount preparations, the three valves of the anterior arteries were isolated together with the base of the arteries. After fixation and washing, tissues of the specimens were immersed in 0.2% trypsin in saline for 30–120 min and the response was stopped by trypsin inhibitor. These specimens were fully rinsed in saline overnight.

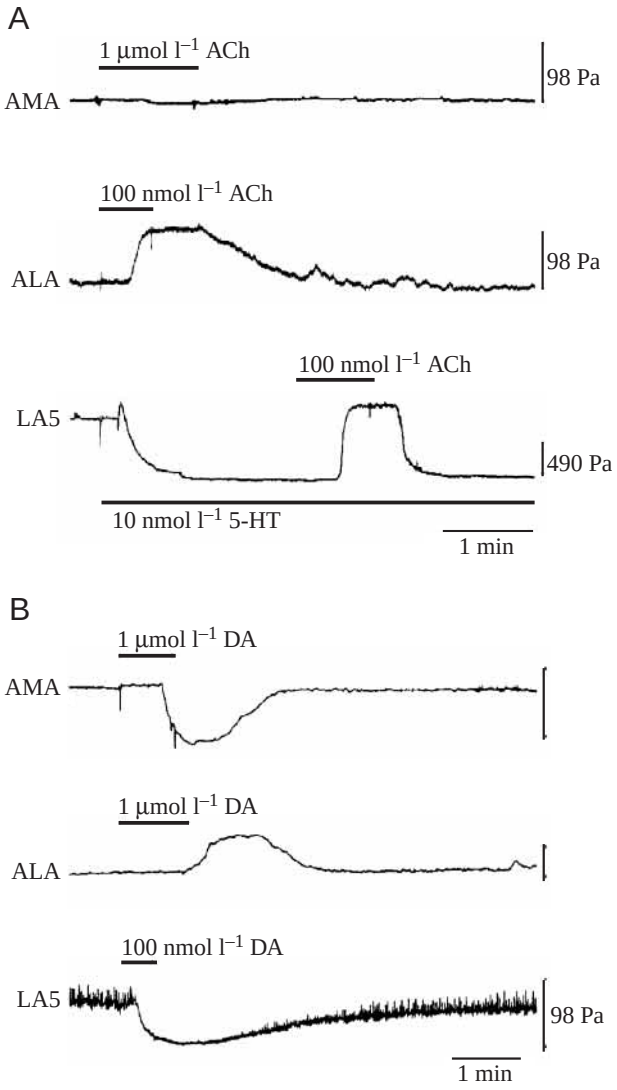


Fig. 2. Effects of (A) acetylcholine (ACh) and (B) dopamine (DA) on arterial pressure in each of the anterior median artery (AMA), anterior lateral artery (ALA) and fifth lateral artery (LA5) preparations without heartbeat (see Materials and methods). Arterial pressure of LA5 was decreased by perfusion with serotonin (5-HT) to accentuate the effects of ACh on pressure of the artery. Bars represent periods of agent application. It was confirmed that an agent solution reached the preparation in approximately 30 s after the start of perfusion.

After treatment with 0.3% H_2O_2 in DW for 45–60 min, the specimens were incubated in the primary antibody diluted in 0.3%-PBT for 40–48 h, in the secondary antibody for 2–4 h and in the PAP complex for 2–4 h. After each incubation, they were fully washed out for 1 h in 0.3%-PBT. The specimens were stained with DAB solutions, dehydrated and cleared in methyl salicylate.

For controls, preparations were processed without the primary antibody to check non-specific staining. Preparations were observed under a microscope (Olympus BX50) and photographed on Kodak Plus X-100 pan films.

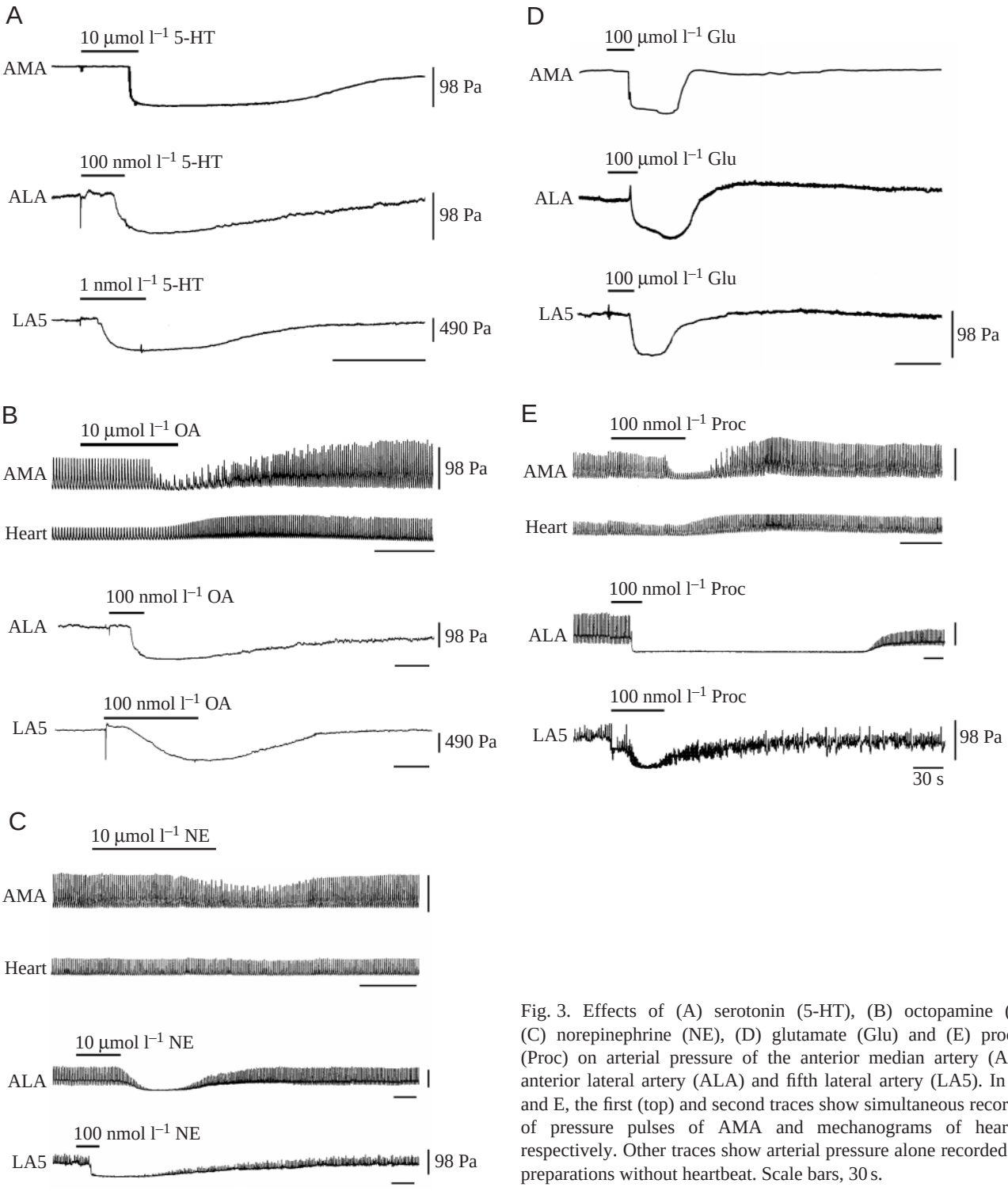


Fig. 3. Effects of (A) serotonin (5-HT), (B) octopamine (OA), (C) norepinephrine (NE), (D) glutamate (Glu) and (E) proctolin (Proc) on arterial pressure of the anterior median artery (AMA), anterior lateral artery (ALA) and fifth lateral artery (LA5). In B, C and E, the first (top) and second traces show simultaneous recordings of pressure pulses of AMA and mechanograms of heartbeat, respectively. Other traces show arterial pressure alone recorded from preparations without heartbeat. Scale bars, 30 s.

Results

Effects of putative neurohormones on the cardioarterial valves

ACh is probably a neurotransmitter for valve inhibitory nerves. Examples may be found in the action of lateral cardiac nerves and in the effect of ACh application on the valves of lateral arteries (Okada et al., 1997). Fig. 2A shows the effects of ACh on pressure in the anterior median artery (AMA), the anterior lateral artery (ALA) and the fifth lateral artery (LA5). Arterial pressure of the AMA showed no response to 1 µmol l^{-1} ACh. The arterial pressure in the ALA was increased by 100 nmol l^{-1} ACh. (The increase of pressure indicates relaxation of the valve muscle.) As the valve in LA5 relaxed previously on ACh application, we first applied 10 nmol l^{-1}

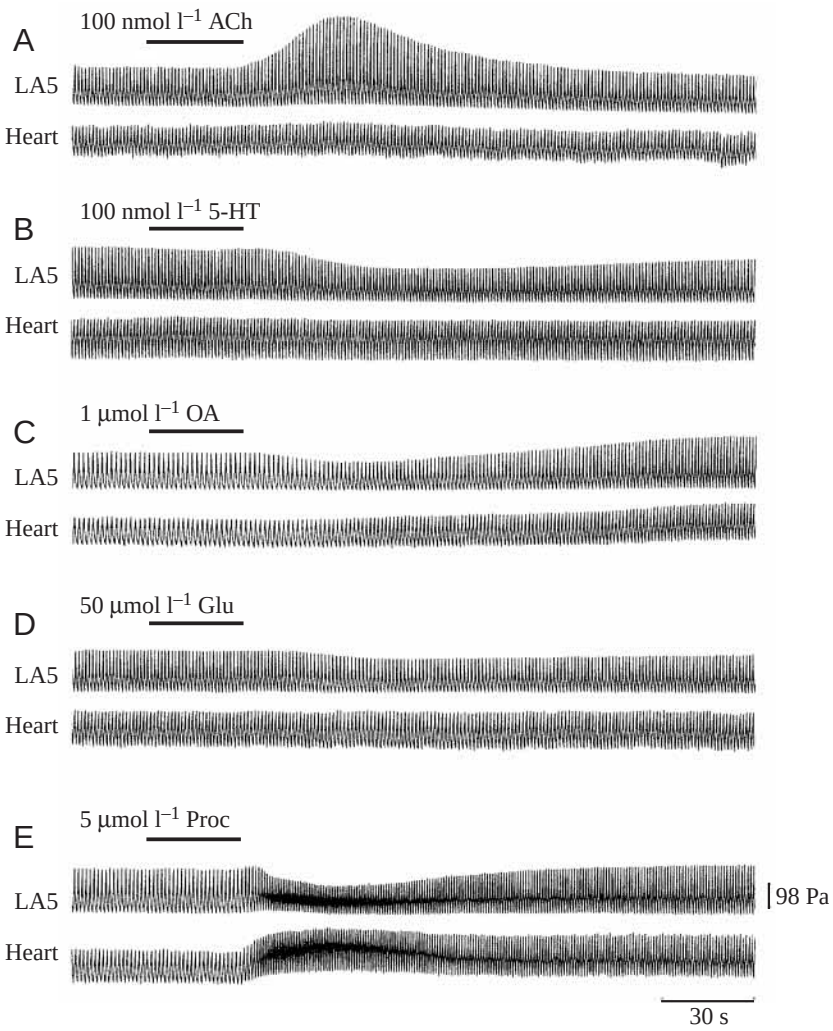


Fig. 4. Simultaneously recorded mechanograms showing effects of (A) acetylcholine (ACh), (B) serotonin (5-HT), (C) octopamine (OA), (D) glutamate (Glu) and (E) proctolin (Proc) on pressure pulses of the fifth lateral artery (LA5) and heartbeat. Bars on each trace indicate the period of agent application.

5-HT to cause light constriction of the valve in Fig. 2A and then applied 100 nmol l^{-1} ACh plus 10 nmol l^{-1} 5-HT. The pressure decreased upon 5-HT treatment, because of contraction of valve muscle (see Fig. 3), and increased when ACh was co-applied.

Fig. 2B shows the effects of dopamine (DA) on artery pressure. Treatment with DA led to a decrease in arterial pressure of the AMA and LA5 but led to an increase in pressure in the ALA. These results suggest that DA exerted constriction in the valve muscle of the AMA and LA5 while relaxing the valve muscle of the ALA.

Serotonin (5-HT) decreased arterial pressure of all three arteries (Fig. 3A). This suggests that 5-HT constricted the valve of the three arteries while the reactivity to 5-HT was diverse among the valves. 10 μmol l^{-1} octopamine (OA) led to a decrease in the amplitude of pressure pulses of the AMA, but this was followed by an increase in pressure pulse amplitude, still in the presence of OA, and an increase in the amplitude of heartbeat throughout (Fig. 3B). These results suggest that OA induces contraction of the valve muscle of the AMA, which first decreased the amplitude of pressure pulses and then excited the heart to overcome the constrictor effects on the valve. 100 nmol l^{-1} OA induced a decrease in the pressure in the ALA and LA5 (Fig. 3B). 0.1–10 μmol l^{-1} norepinephrine (NE) decreased the pressure of all three arteries (Fig. 3C); however, NE showed no obvious effect on the heartbeat. 100 μmol l^{-1} glutamate (Glu) decreased the pressure of all three arteries (Fig. 3D). The effects of 100 nmol l^{-1} proctolin (Proc) on the amplitude of pressure pulses of the AMA and on the heartbeat recorded simultaneously (Fig. 3E) were very similar to the effects of OA in Fig. 3B. Proc apparently decreased the amplitude of pressure pulses of the AMA first. Proc also decreased the pressure of the ALA and LA5 (Fig. 3E) These results suggest that OA, NE, Glu and Proc induced constriction of all three valves. These effects of the agents are summarised in Table 1.

Table 1. *Effects of neurohormones on the cardioarterial valve of the arteries and the heart*

	ACh	5-HT	OA	DA	NE	GABA	Glu	Proc
AMA	No	E	E	E	E	–	E	E
ALA	I	E	E	I	E	–	E	E
LA5	I	E	E	E	E	–	E	E
Heart	E	E	E	I	I	I	E*	E

AMA, anterior median artery; ALA, anterior lateral artery; LA5, fifth lateral artery; ACh, acetylcholine; 5-HT, serotonin; OA, octopamine; DA, dopamine; NE, norepinephrine; GABA, γ-aminobutyric acid; Glu, glutamate; Proc, proctolin; E, excitatory (constrictor) effect; I, inhibitory (dilator) effect; No, no effect; –, uncertain effect.

*Data taken from Tanaka et al. (1992).

Effects of putative neurohormones on cardiac outflow

In order to observe the effects of putative neurohormones on both the heart and the valve of LA5, we recorded the mechanogram of the heartbeat and the pressure pulse of the artery simultaneously (Fig. 4). Fig. 5 shows dose–response curves. ACh increased the amplitude of pressure pulses (Fig. 4A; filled circles in Fig. 5A). The threshold for this valve-dilating effect was between 100 pmol l^{-1} and 1 nmol l^{-1}. ACh also increased the heart rate (open squares in Fig. 5A) and contraction force (open triangles in Fig. 5A). The threshold for these effects was between 100 nmol l^{-1} and 1 μmol l^{-1}. 5-HT, OA and Proc decreased the amplitude of pressure pulses

(Figs 4B,C,E, 5B,C,E; see Fig. 3A,B,E) while they activated the heart. The thresholds for the valve-constricting effects on the pressure and for the positive inotropic and chronotropic effects on the heart were approximately the same; concentrations of 1–10 nmol l^{-1} for 5-HT, 10–100 nmol l^{-1} for OA and approximately 100 nmol l^{-1} for Proc. Glu decreased heart rate (Fig. 5D). High concentrations of Glu (>200 μmol l^{-1}) induced systolic arrest in this preparation. In this case, the data were measured at the point just before systolic arrest occurred (cf. Yazawa et al., 1998). The amplitude of pressure pulses was decreased by application of Glu (Figs 4D, 5D; see Fig. 3D). The threshold concentration of Glu for decrease of pressure pulses was approximately 20 μmol l^{-1}.

Fig. 6 shows the effects of stimulation of the fifth lateral cardiac nerve (LCN5) on the pressure pulses in LA5 during perfusion of the valve with monoamines that induce constriction. When LCN5 was stimulated at 1–10 Hz, the amplitude of pressure pulses increased in a frequency-dependent manner in SW (control). During application of 5-HT (100 nmol l^{-1}) or OA (1 μmol l^{-1}), the frequency of pressure pulses (i.e. heart rate) increased by 24% for 5-HT and by 29% for OA because of the excitatory effect of the monoamines, but the amplitudes of pressure pulses were decreased because of the constricting effect of the monoamines on the valve muscles (cf. Figs 4B,C, 5B,C). The effect of stimulation of LCN5 also appeared as in SW. These results indicate that the constricting effects of the monoamines were surpassed by the dilating effects of the inhibitory valve nerves.

Effects of valve constrictor agents on the membrane potential of the valve muscle cells of the AMA

5-HT, OA, NE, Proc and Glu decreased the arterial pressure of the AMA and the ALA (Fig. 3). These results indicate that the agents constricted the valves of the arteries. However, high concentrations of 5-HT, OA, NE and Proc have no effect on membrane potentials of valve muscle cells of the AMA. Among the substances that induce valve constriction, only Glu depolarized valve muscle cells of the AMA in a dose-dependent manner. Fig. 7A shows the effects of bath-applied Glu on the membrane potential of a valve muscle cell. Glu exerts depolarizing effects in a dose-dependent manner; 50 μmol l^{-1} Glu exerts slightly depolarizing effects, while the depolarization reached –18 mV in response to 1 mmol l^{-1} Glu. The depolarizing responses were dose dependent (Fig. 7B). The threshold concentration of Glu for the responses was estimated at 10–50 μmol l^{-1}.

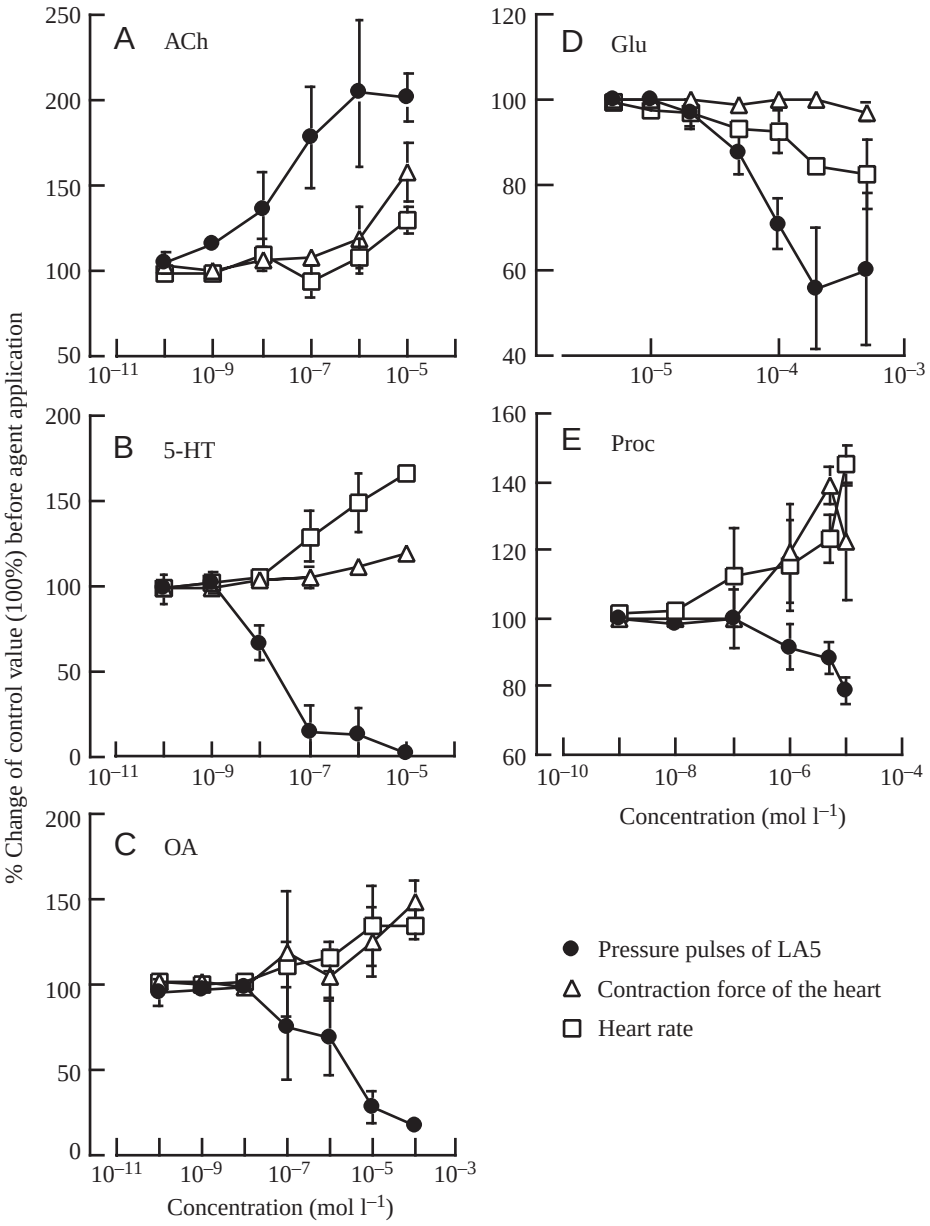


Fig. 5. Dose–response curves showing the relationship between cardioexcitatory substances – (A) acetylcholine (ACh), (B) serotonin (5-HT), (C) octopamine (OA), (D) glutamate (Glu) and (E) proctolin (Proc) – and amplitude of pressure pulses of the fifth lateral artery (LA5; filled circle), heart rate (open square) and contraction force of the heart (open triangle). All data (mean ± S.D., *N*=3) were obtained from one preparation for three times applications per one dose. Each of the five graphs was obtained from a different specimen. The values plotted for pressure pulses, heart rate and contraction force were obtained at the maximum change of the amplitude of pressure pulses.

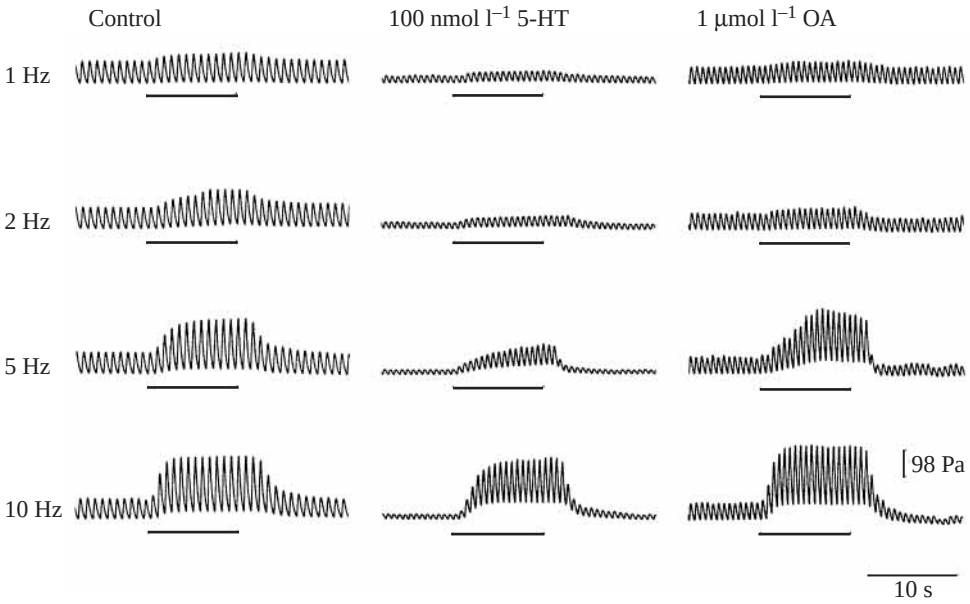


Fig. 6. Effects of stimulation of the fifth lateral cardiac nerve (LCN5) on pressure pulses recorded from the fifth lateral artery (LA5) during perfusion of SW (control), serotonin (5-HT) and octopamine (OA). These data were obtained from one preparation. Bars show the periods of stimulation. Frequencies of stimulation are shown at the beginning of the traces in the left column.

Effects of glutamatergic agonists and antagonists on the membrane potential of valve muscle cells of the AMA

The glutamatergic agonists kainate (1 mmol l^{-1}) and quisqualate (100 nmol l^{-1}) produced depolarizing effects on valve muscle cells of the AMA (Fig. 7C). The glutamatergic antagonist MK-801 (1 mmol l^{-1}) blocked EJPs in the valve muscle, evoked by stimulation of the valve excitatory nerve (Fig. 8A). Effects of the glutamatergic antagonist JSTX on EJPs are shown in Fig. 8B. The amplitude of each EJP in a series of superimposed EJPs varied in spite of constant stimuli (cf. Kihara et al., 1985). The amplitude of EJPs before treatment with JSTX (5 μmol l^{-1}) was 4.24±1.48 mV (mean ± S.D., *N*=15) and the amplitude was reduced to 2.19±0.80 mV (*N*=15) during the treatment. The effects of MK-801 were reversible, whereas the effects of JSTX did not reverse for at least one hour after the start of washing with saline.

Immunocytochemistry

Fig. 9 shows micrographs obtained from cross sections of the anterior cardiac nerve (ACN) and schematic drawings. These two sections are neighbouring; Fig. 9A is treated with anti-GABA antibody and Fig. 9B is treated with anti-Glu antibody. It has been suggested from pharmacological experiments that, among the five axons in the ACN, the two cardioacceleratory axons (Tanaka et al., 1992) and the valve inhibitory axon (Okada et al., 1997) are cholinergic, while the cardioinhibitory axon is GABAergic (Tanaka et al., 1992). In Fig. 9A, GABA-like immunoreactivity is seen in one of five axons, which is probably the cardioinhibitory axon. In Fig. 9B, one axon shows strong Glu-like immunoreactivity and the other shows weak Glu-like immunoreactivity, the latter showing GABA-like immunoreactivity in Fig. 9A. Therefore, it is likely that the cardioinhibitory axon shows weak crossreactivity with the Glu antibody. The axon that has strong Glu-like immunoreactivity could be the valve excitatory axon, because the other three axons (the two cardioacceleratory axons and the valve inhibitory axon) are known to be cholinergic.

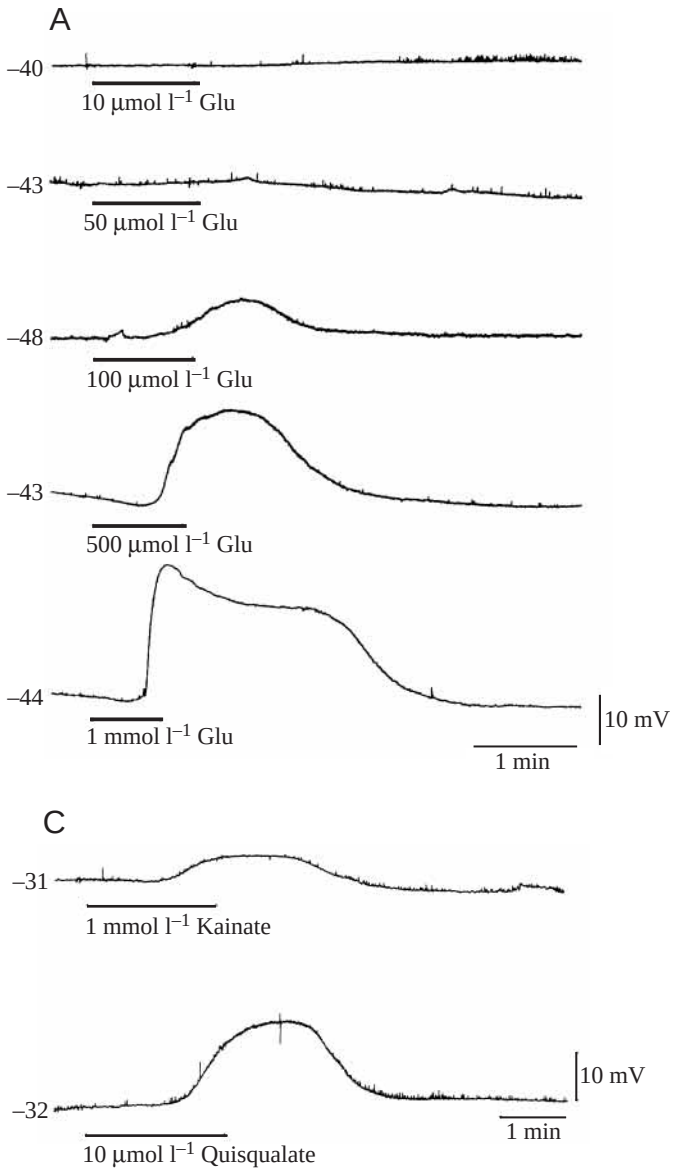
We observed that the Glu-like immunoreactive axon ran through the anterior cardiac nerve and ramified over the valve flaps to run along the valve muscle fibres in a whole-mount preparation. The micrographs A and B in Fig. 10 were taken of the parts indicated by the squares A and B of the valves of the AMA in the illustration. Immunoreactive axonal processes and varicosities are seen there.

Fig. 11 summarises the anatomical, pharmacological and immunocytochemical results of studies of the innervation of the cardiovascular system in the anterior half of the heart of *Bathynomus*. Cholinergic, GABAergic and glutamatergic axons are coloured red, blue and green, respectively. On the inner wall of the heart, glutamatergic neuronal cell bodies and their axons form a cardiac ganglion (CG). The cardiac ganglion is innervated by cholinergic (CA1 and CA2) axons and the GABAergic (CI) axon. The valve of the AMA is innervated by a pair of glutamatergic excitatory axons. The valve of the right or left ALA is innervated by a pair of cholinergic inhibitory axons and a pair of excitatory axons. The valve of the first lateral artery (LA1) is innervated by the cholinergic dual axon nerve, the first lateral cardiac nerve (LCN1). The valve of the second lateral artery (LA2) is innervated by the cholinergic single axon nerve, the second lateral cardiac nerve (LCN2).

Discussion

Neurohormonal regulation of the distribution of haemolymph to arteries

We examined the effects of putative crustacean neurohormones on perfusate pressure in the AMA, the ALA and LA5 (as a representative of all lateral arteries) of



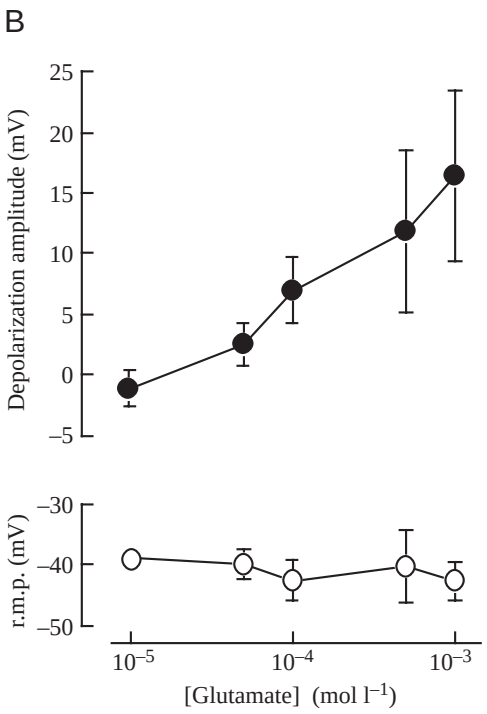


Fig. 7. (A) Effects of glutamate (Glu) on the membrane potential of valve muscle cells in the anterior median artery (AMA). All data were obtained from the same muscle cell. Bars on each trace indicate the period of Glu application. Concentrations of Glu are indicated under the bars. Numerals at the beginning of traces represent membrane potential (mV) in this and following figures. (B) The dose–response relationship between Glu concentrations and depolarization of valve muscle cells of AMA (filled circle). Membrane potentials were obtained from four animals. Resting membrane potentials (r.m.p.; open circle) were measured before doses. Values represent mean ± S.D. (N=4–6). (C) Effects of glutamatergic agonists kainate and quisqualate on the membrane potential of valve muscle cells in AMA. Bars indicate the periods of agonist perfusion.

Bathynomus in order to clarify the pharmacological properties of valve muscle cells of the arteries (Table 1).

ACh may be a neurotransmitter for the cardioacceleratory axons (CA1 and CA2; Tanaka et al., 1992) and for the valve inhibitory axons (Okada et al., 1997). ACh applied to the bath exerted excitatory effects on the heart, while it exerted a valve-dilating effect on LA5 (Fig. 5). The threshold concentration for the effect of ACh (1 nmol l^{-1}) on pressure pulses in LA5 was the same as that observed by Okada et al. (1997) for membrane hyperpolarization of valve muscle cells of LA5. Therefore, the increase in the pressure pulse amplitude is attributable to dilation of the valve caused by hyperpolarization of valve muscle cells.

5-HT, OA, DA and Proc are known to be stored in the pericardial organ in decapods. Dalaleu (1970) observed the pericardial organ in the isopod crustaceans *Porcellio dilatatus*, *Helleria brevicornis* and *Ligia oceanica*. In *Bathynomus*, we also observed a pair of pericardial organ-like structures, including 12 cell bodies stained by methylene blue and neutral red at the dorsal wall of the pericardial cavity (not shown). The cells that contained biogenic amines were stained by neutral red (Stuart et al., 1974). Thus, *Bathynomus* may have a pericardial organ that contains biogenic amines.

5-HT, OA and Proc had a cardioexcitatory effect in *Bathynomus* (Tanaka et al., 1992). 5-HT (>10 nmol l^{-1}), OA(>100 nmol l^{-1}) and Proc (>1 μmol l^{-1}) have strongly constricting effects on the valve of LA5 (Figs 4, 5). The values may show rather higher than their simple effects on the valve because they were recognized as concentrations for overcoming their excitatory effects on the heart. Mean concentrations of normally circulating 5-HT and OA were measured to be 1.4±1.8 nmol l^{-1} and 2.3±1.3 nmol l^{-1}, respectively, in the lobster *H. americanus* (Livingstone et al., 1980). Sneddon et al. (2000) reported that circulating levels of

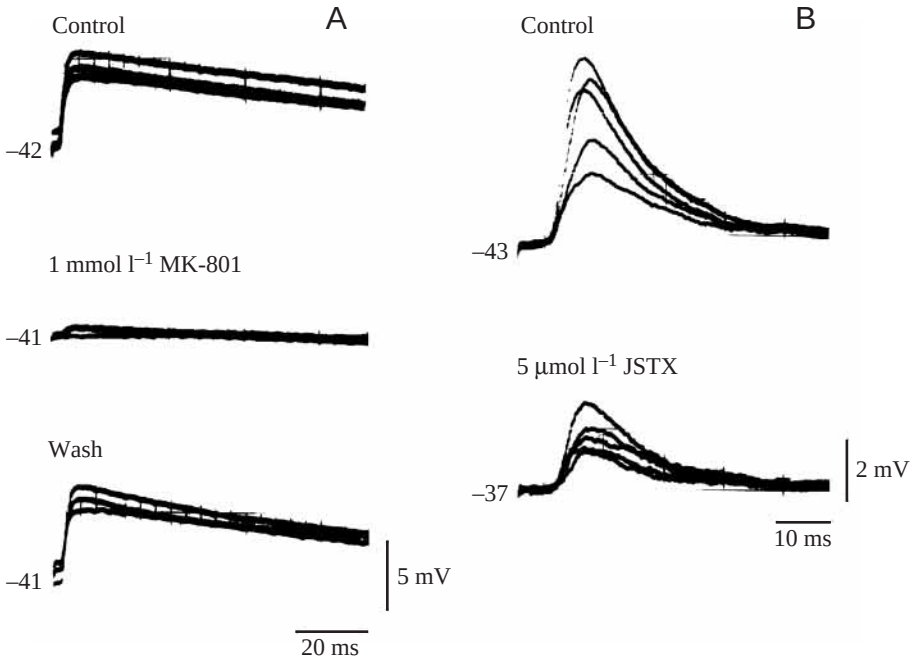


Fig. 8. Effects of the glutamatergic antagonists MK-801 and Joro spider toxin (JSTX) on excitatory junctional potentials (EJPs) evoked on valve muscle cells of the anterior median artery (AMA) by stimuli applied to the valve excitatory nerve (VE). Three (A) and five (B) sweeps triggered by stimulus pulses were superimposed. (A) 3 min after the onset of perfusion with MK-801 (1 mmol l^{-1}), EJPs were almost abolished. After washing with saline, EJPs recovered. (B) 25 min after bath application of JSTX (concentration was calculated to be 5 μmol l^{-1}), the amplitude of each of five EJPs was diminished by more than half. The effect of JSTX did not reverse within one hour.

5-HT and OA in the shore crab *Carcinus maenas* were approximately 5.7 nmol l^{-1} and 8.2 nmol l^{-1}, respectively. However, as these concentrations were measured from systemic circulation haemolymph, one may not exclude the possibility that the concentrations of the pericardial hormones at the cardioarterial valves in the heart are higher than these values. It remains to be determined what circulatory concentrations of the monoamines may be measured inside the heart, where the cardioarterial valves are located in *Bathynomus*.

DA and NE are reported to produce cardioinhibitory activity in *Bathynomus* (Tanaka et al., 1992). We generally could not observe their effects on arterial pressure pulses because the threshold concentrations of either DA or NE for inhibitory effects on the heartbeat were lower than that for inhibitory (dilating) effects on the valve. NE was detected in the cardiac ganglion of the lobster *H. americanus* (Occor and Berlind, 1983), although it is not known to act as a neurohormone in decapods. Valve-constricting effects of NE at a relatively high concentration (Fig. 3C) might show crossreaction of NE to receptors for other catecholamines such as DA and OA.

Glu, a neurotransmitter candidate for cardiac ganglionic cells of *Bathynomus* (Yazawa et al., 1998), has been reported to accelerate the isolated *Bathynomus* heart (Tanaka et al., 1992). Thus, we cite the results of experiments by Tanaka et al. (1992) as an excitatory effect (E) in Table 1. However, heart rate was reduced in the present study (Fig. 5). In isolated heart preparations in which all arteries except for a measuring artery were tied off, the inhibitory effects of Glu on heartbeat may reflect overloading with elevated intracardiac pressure. The effects of Glu on the valve muscle cells of the measuring artery will be discussed later. Although GABA is supposed to be a neurotransmitter of the valve nerve in decapods, up to 100 μmol l^{-1} of GABA produced no apparent effect on arterial pressure of the arteries (Table 1). It is unlikely that a GABAergic mechanism is involved in regulation of the *Bathynomus* cardioarterial valves.

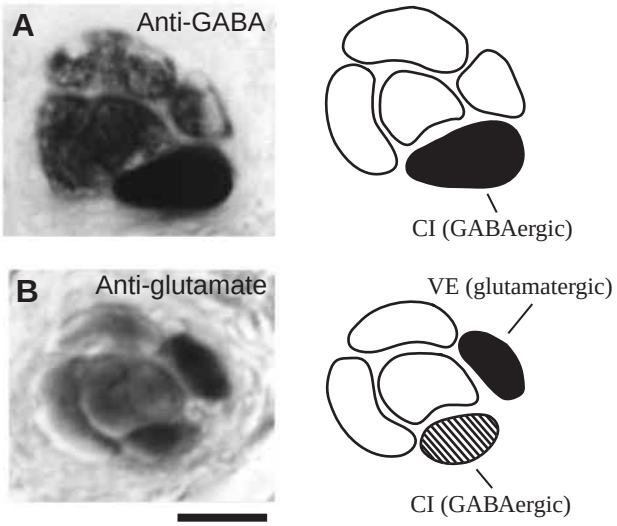


Fig. 9. Micrographs (left) and schematic drawings (right) of cross sections of the anterior cardiac nerve (ACN) treated with (A) anti-GABA (γ-aminobutyric acid) antibody or (B) anti-Glu (glutamate) antibody. A and B are neighbouring sections. In A, one GABA-like immunoreactive axon profile is seen. In B, one strong and one weak (the hatched axon in the schematic drawing indicates the weak profile) Glu-like immunoreactive axon profile is seen. Scale bar, 5 μm. CI, cardioinhibitory nerve; VE, valve excitatory nerve.

The valves of the arteries (AMA, ALA and LA5) responded to the neurohormones in their own ways, changing the cardiac outflow into the arteries (Table 1). These neurohormones can generate a variety of haemodynamics by their effects on both the heart and valves. For example, DA inhibits heartbeat and

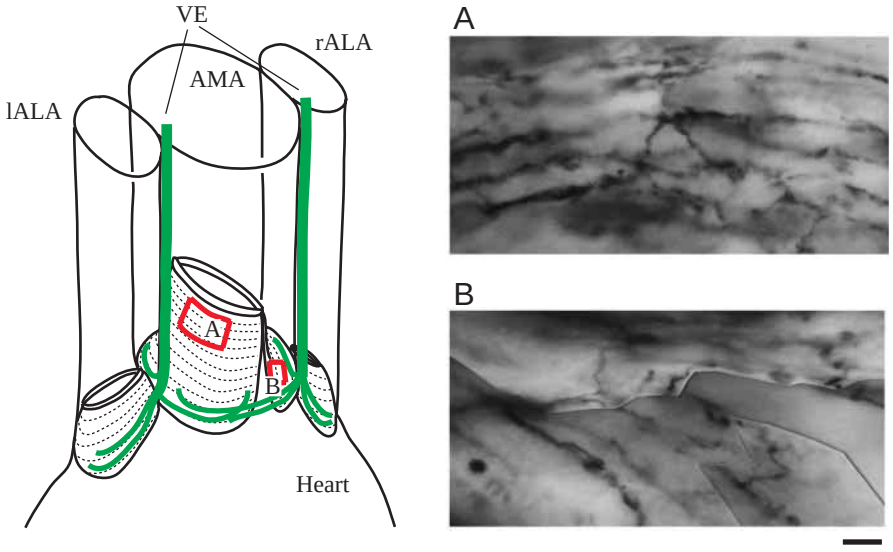


Fig. 10. Micrographs of immunoreactive fibres and varicosity-like structures, which were revealed with anti-Glu (glutamate) antibody in the valve of the anterior median artery (AMA). The location of micrographs A and B is indicated in the schematic drawing. Scale bar, 10 μm. VE, valve excitatory nerve; rALA, right anterior lateral artery; lALA, left anterior lateral artery.

constricts the valves of the AMA and LA5 but relaxes the valve of the ALA. This mode may allow haemolymph flow to be concentrated on muscles of the cephalon, which are responsible for movement of the oral appendages. The crustacean FLRFamide-like peptides are known to initiate pyloric and gastric rhythms in *Cancer borealis* (Weimann et al., 1993). They decreased cardiac output in *Cancer magister* but supplied a greater part of the cardiac output to the hepatic artery, extending to the digestive organs (McGow and McMahon, 1995). These observations support the concept that neurohormonal control of the valve, for the arterial distribution of cardiac outflow, is conserved in general for crustaceans.

Evidence for the neural regulation of haemolymph distribution, according to exercise-induced demands of organs, has been reported only in the isopod *Bathynomus*. When swimmerets beat strongly, impulse rate always increased in the axon of LCN5 to the valve of LA5, which supplies haemolymph to swimmeret muscles. Impulse rate decreased in the first lateral cardiac nerve, the dilator of the valve of the first lateral artery, which supplies haemolymph to walking legs (Okada and Kuwasawa, 1995). We observed that when impulse rates of valve inhibitory and valve excitatory axons increased during movements of the oral appendages, the haemolymph supply was increased to the muscles of the oral appendages through the ALA.

Hormonal regulation in association with neural regulation may put in operation a complex mode of haemolymph distribution. In fact, stimulation of the LCN5, a cholinergic valve inhibitory nerve, augmented pressure pulses of LA5 even when the valve muscles were strongly constricted by 5-HT or OA (Fig. 6). Wilkens et al. (1996) observed that, in the lobster *H. americanus*, stimulation of the inhibitory nerve to the valve of the sternal artery increased the outflow of the artery, which had decreased by the combined application of OA and Proc. 5-HT, OA, Glu and Proc accelerate heartbeat and simultaneously constrict all valves, which causes an extraordinary increase of intracardiac pressure. During such a state of increase in intracardiac pressure, cardiac outflow may be correlated on a

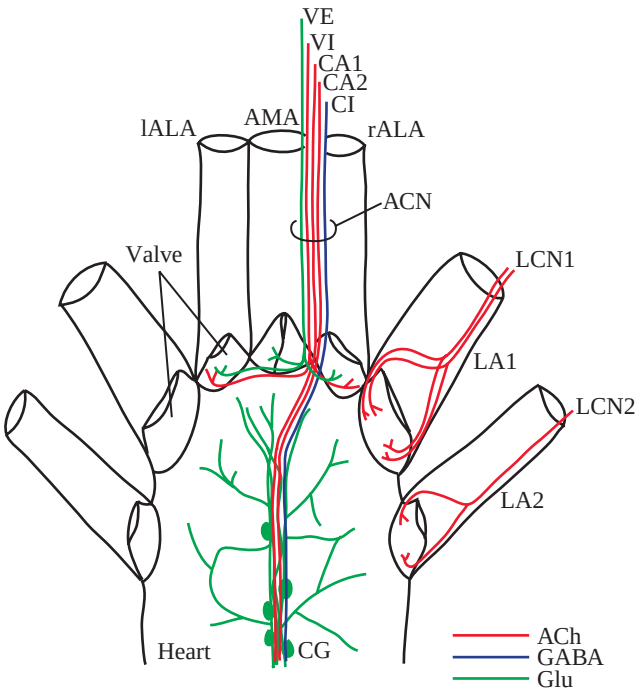


Fig. 11. Schematic drawing of anterior cardiac nerves (ACN) and valve nerves in the anterior part of the heart of *Bathynomus*. An ACN contains five axons; glutamatergic valve excitatory (VE; green), cholinergic valve inhibitory (VI) and cardioacceleratory (CA1 and CA2) (red), and GABAergic cardioinhibitory (CI; blue). The first lateral cardiac nerve (LCN1) contains two cholinergic axons, and LCN2 contains one cholinergic axon. The cell bodies and axons (green) in the heart are the cell bodies and axons of glutamatergic cardiac ganglionic neurons (CG). AMA, anterior median artery; lALA, left anterior lateral artery; rALA, right anterior lateral artery; LA, lateral artery; ACh, acetylcholine; GABA, γ-aminobutyric acid; Glu, glutamate.

particular vascular bed. For instance, the valve of a particular artery may be opened by impulses in an inhibitory axon of a valve nerve. Thus, haemolymph distribution may be coordinated with behavioural demands. However, this would not apply to the valve of the AMA, which lacks inhibitory innervation.

Effects of monoamines and proctolin on the membrane potential of valve muscle cells

In *Panulirus japonicus*, 5-HT often hyperpolarized muscle cells of both anterior and posterior cardioarterial valves (Kuramoto and Ebara, 1984). OA (100 nmol l^{-1}) hyperpolarized muscle cells in the anterior valve while depolarizing the posterior valve (Kuramoto and Ebara, 1984). Proc depolarized muscle cells of both the valves (Kuramoto and Ebara, 1984). In *Homarus americanus*, 5-HT (1 μmol l^{-1}) and OA (1 μmol l^{-1}) depolarized valve muscle cells of the posterior artery (Kuramoto et al., 1992). In the present study, when the four hypotensive agents, 5-HT, OA, NE and Proc, were applied to the valve of the AMA, even at suprathreshold concentrations for arterial pressure, they never caused changes of membrane potential. We could not detect their effects on the membrane potential of muscle cells in any valve specimens, whereas arterial pressure was evidently decreased by contraction of valve muscles caused by these agents (Fig. 3). 5-HT induced contraction of the dactyl opener muscle in the propodite segment of the walking leg without membrane depolarization in the lobster *H. americanus* (Kravitz et al., 1980, Glusman and Kravitz 1982). The non-voltage-dependent mechanisms for contraction of the valve remain to be examined with pharmacological tools for intracellular calcium behaviour.

A neurotransmitter for the valve excitatory nerve

Only Glu among valve constrictor neurohormones caused relatively slow and long-lasting depolarization of valve muscle cells in the AMA in a dose-dependent manner (Fig. 7A). Induction of depolarization is an essential character of a neurotransmitter candidate for the valve excitatory nerve because stimulation of the nerve induced EJPs in valve muscle cells (Fig. 8; Kihara et al., 1985). Glu is known as an excitatory neurotransmitter at neuromuscular junctions of skeletal muscles in crustaceans (reviewed by Shinozaki, 1980; Atwood, 1982). Quisqualate and kainate, which are known as agonists of glutamatergic receptors at neuromuscular junctions of skeletal muscles in some decapods (Shinozaki, 1988), depolarized the membrane potential of valve muscle cells of the AMA (Fig. 7C). The compounds mimicked glutamatergic actions in the *Bathynomus* myocardium (Yazawa et al., 1998). JSTX, known as an irreversible antagonist for glutamatergic receptors in decapod neuromuscular junctions (Kawai et al., 1982), decreased the amplitude of EJPs evoked by valve excitatory nerve stimulation (Fig. 8B). Moreover, MK-801 is known to be a channel blocker of the *N*-methyl-D-aspartate (NMDA) receptor in mammals, and it blocked the EJPs (Fig. 8A).

Two kinds of immunoreactivity against anti-Glu antibody were seen in cross sections of the anterior cardiac nerve (Fig. 9). Of the two Glu-like immunoreactive axons, one had weak reactivity and the other had strong reactivity. The former, but not the latter, axon showed strong GABA-like immunoreactivity against anti-GABA antibody in a neighbouring cross section. The anti-Glu antibody has 3% crossreactivity to GABA compared with Glu, so we conclude that the weak Glu-like immunoreactive axon is a GABAergic cardioinhibitor and the other axon that has stronger Glu-like immunoreactivity would be the valve excitatory axon. Immunocytochemistry of whole-mount preparations of the valve of the AMA revealed that peripheral processes of the valve excitatory nerve had Glu-like immunoreactivity (Fig. 10). One axon in the anterior cadiac nerve and its branches on valves of the anterior arteries showed glyoxylic acid-induced fluorescence (Kihara et al., 1985). The fluorescence might be due to Glu because many amino acids, as well as monoamines, showed fluorescence with glyoxilic acid (Keenan and Koopowitz, 1981). From the pharmacological and immunocytochemical analyses, we may conclude that Glu is a neurotransmitter for the valve excitatory nerve.

Transmitters for neural elements in the cardiac motor nervous system in *Bathynomus* have now been proposed for the whole system (Fig. 11). There are 12 glutamatergic cardiac ganglionic cells (Yazawa et al., 1998), two pairs of cholinergic cardioacceleratory nerves (Tanaka et al., 1992), a pair of GABAergic cardioinhibitory nerves (Tanaka et al., 1992), six pairs of cholinergic inhibitory valve nerves (a pair of valve inhibitory nerves and five pairs of the lateral cardiac nerves) (Okada et al., 1992) and, as shown here, a pair of glutamatergic valve excitatory nerves.

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