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Cholinergic Inhibitory Innervation of the Cardioarterial Valves in the Isopod *Bathynomus doederleini*

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ABSTRACT—In the isopod *Bathynomus doederleini*, the cardioarterial valves of all five pairs of lateral arteries and the pair of anterior lateral arteries are innervated by inhibitory (dilator) nerves which consist of one or two axons arising from the central nervous system. Stimulation of the valve dilator nerves produced inhibitory junctional potentials (IJPs) in valve muscle cells which arose one-to-one in response to stimulus pulses. Acetylcholine (ACh) hyperpolarized muscle cells of the valves. Both the IJPs and ACh-induced hyperpolarization brought about an increase of haemolymph pressure in the arteries, through relaxation of valve muscles. The muscarinic agonists, muscarine, carbamylcholine and arecoline, mimicked ACh-induced hyperpolarizing responses of the valve muscle cells. Atropine and methylxylocholine antagonized both the IJPs and ACh-induced hyperpolarizing potentials, while *d*-tubocurarine did not antagonize IJPs. These results indicate that ACh may be the transmitter for the valve dilator nerves. IJPs did not invert in Cl^--free saline. Amplitude of IJPs increased in low K^+ salines, and decreased in high K^+ salines. It is likely that IJPs are mediated predominantly by K^+ ions. This could be the first case of cholinergic inhibitory transmission at neuromuscular junctions in crustaceans.

INTRODUCTION

It is well known that in molluscs and vertebrates blood distribution to the destinations of the arteries is accomplished by neural and humoral control of the vasomotor system. A ubiquitous feature of most crustacean circulatory systems is the absence of arterial musculature (reviewed by Maynard, 1960), except for the dorsal abdominal artery. In *Panulirus interruptus* (Burnett, 1984), *Sicyonia ingentis* (Martin *et al.*, 1989) and *Homarus americanus* (Wilkens *et al.*, 1997), the dorsal abdominal artery has striated musclature in its walls. Cardioarterial valves are located at the junctions between the heart and individual arteries (Alexandrowicz, 1932, 1934, 1952). The dorsal abdominal artery in *Homarus americanus* lacks a cardioarterial valve, but arterial valves are located at the origin of each of the major paired lateral segmental branches (Wilkens and Davidson, 1995; Wilkens, 1997). All these valves receive innervation from the central nervous system (Kihara and Kuwasawa, 1984; reviewed by McMahon and Burnett, 1990; Kuramoto *et al.*, 1992; Wilkens and Davidson, 1995). Haemolymph flow in the arteries and haemolymph distribution from the heart to the arteries are controlled by the cardioarterial valves (Kihara and Kuwasawa, 1984; Kihara *et al.*, 1985; Fujiwara-Tsukamoto *et al.*, 1992; Kuramoto *et al.*, 1995; Okada and Kuwasawa, 1995) and the arterial valves (Wilkens and Davidson, 1995; Wilkens, 1997).

In *Bathynomus*, three anterior arteries (AAs) and five pairs of lateral arteries (LA1-5) arise from the heart. The three AAs are an anterior median artery (AMA) and a pair of anterior lateral arteries (ALAs). All the cardioarterial valves are innervated by their own valve nerves from the central ganglia. The cardioarterial valves of the ALAs receive dual innervation by excitatory and inhibitory nerves, the valves of the LAs receive single innervation by inhibitory nerves, and the valve of the AMA receives single innervation by excitatory nerves. The excitatory valve nerve brings about contraction of a pair of valve flaps, resulting in a decrease of arterial haemolymph flow (Kihara and Kuwasawa, 1984; Kihara *et al.*, 1985) in AMA and ALAs. On the other hand, the inhibitory valve nerves cause expansion of the bore of the valve, resulting from dilation of the valve flaps, and thus produce an increase of haemolymph flow in ALAs and LAs (Kihara and Kuwasawa, 1984; Kihara *et al.*, 1985; Fujiwara and Kuwasawa, 1987; Fujiwara-Tsukamoto *et al.*, 1992).

In this study, we characterize the ionic mechanisms of inhibitory junctional potentials (IJPs), and reveal cholinergic characteristics of the neurotransmitter for the valve dilator

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nerves. Preliminary reports on pharmacological aspects of control of the valves have appeared elsewhere in abstract form (Kihara and Kuwasawa, 1985; Fujiwara and Kuwasawa, 1987; Okada and Kuwasawa, 1993).

MATERIALS AND METHODS

Specimens of *Bathynomus doederleini*, 9-15 cm in body length, were collected and kept in a laboratory aquarium as described previously (Okada and Kuwasawa, 1995).

Preparations

For intracellular recording from valve muscle cells, the basal part of an artery, containing the pair of flaps which constitute the cardioarterial valve, was severed from the heart, and dissected out together with the valve nerve. The specimens were carefully incised along the long axis of the artery, and pinned inside out to the Sylgard-lined bottom of an experimental bath (0.5 ml) in order to expose the valve.

Intact valves were used for pressure recording from arteries. The arteries were perfused with solutions through a cannula whose tip was positioned toward the beginning of the artery. Arterial haemolymph flow was recorded with a pressure transducer (Gould, P-50). The probe of the transducer was connected to a polyethylene cannula (0.25 mm in inside diameter) whose tip was inserted into the artery.

Artificial sea water (ASW) was routinely applied to the bath through a temperature-controlling bath unit (Model CTC-100, Aqua, Tokyo, Japan) by gravity-feeding, and suctioned for removal. ASW and drug-containing ASW were superfused on preparations in the bath, by means of turning the cock of a three-way valve at flow rates of 0.7–2.1 ml/min for intracellular recording, and of 2.2–3.2 ml/min for pressure recording. Temperature in the chamber was kept at 17-20°C.

Electrophysiology

Stimulus pulses (0.2-0.5 ms in duration) were applied to the cut-stump of the valve nerve through a suction electrode containing an Ag-AgCl wire. Intracellular potentials of the valve muscle cells were recorded with glass microelectrodes filled with 3 M KCl (tip resistance, 10–40 MΩ), using a conventional electrophysiological technique. In experiments using Cl^--free salines, an Ag-AgCl reference electrode communicated with the bath via a 3 M KCl reservoir and an agar bridge (Kuwasawa *et al.*, 1987).

ACh was applied to the valve muscle, using an iontophoretic glass micropipette filled with 1 M ACh (tip resistance, 15-80 MΩ). Positive current pulses (15–150 nA, 40–200 ms in duration) were applied to the micropipette. A negative retaining current of about 5 nA was continuously applied to the micropipette. The tip of an ACh-filled pipette was positioned as close to the recording microelectrode as possible.

Solutions

Ionic compositions of various kinds of salines are shown in Table 1. The ionic composition of the ASW was almost the same as that of Yazawa and Kuwasawa (1984). Low K^+ salines were prepared by reducing the concentration of KCl. High K^+ salines were prepared by replacing NaCl with KCl. For making solutions at various concentrations of Na^+, Tris (hydroxymethyl) aminomethane hydrochloride was substituted for NaCl. Cl^--deficient salines were prepared by replacing Cl^- with propionate and sulfate. Tris (hydroxymethyl) aminomethane was added to adjust pH at 7.5–7.8.

Chemicals

The following chemicals were used: acetylcholine chloride, γ-aminobutyric acid, atropine sulfate monohydrate, eserine sulfate, nicotine tartrate, ouabain, picrotoxin, *d*-tubocurarine chloride (Wako Pure Chemical), arecoline hydrobromide, bicuculline methobromide, carbamylcholine chloride, *dl*-muscarine chloride (Sigma), methylxylocholine chloride (also called β-methyl TM10, a gift from Smith, Kline & French Laboratories, Philadelphia, Pennsylvania).

RESULTS

Effects of ACh and cholinergic agonists on the valve muscle

Intracellular potentials were recorded from muscle cells of the cardioarterial valves. Resting membrane potentials of the valves of LA5 ranged from –25.0 to –58.0 mV (–37.6 ± 8.0 mV, mean ± SD, N = 44). Effects of ACh on muscle cells of all the cardioarterial valves, i.e. the valves of the AMA, ALAs and LAs are shown in Fig. 1. Bath-applied ACh (10^{-7} M) hyperpolarized muscle cells of the valves of the ALA and LA1-5 by 20-30 mV. It was noticeable that the valve muscle cells of the

Table 1. Ionic composition of solutions (mM)

		Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Tris-HCl	propionate	SO_4^{2-}
ASW		526.0	11.0	18.0	23.5	626.7	6.7	–	–
Low K^+	(1)	526.0	1.0	18.0	23.5	616.7	6.7	–	–
	(2)	526.0	2.0	18.0	23.5	617.7	6.7	–	–
	(3)	526.0	5.0	18.0	23.5	620.7	6.7	–	–
High K^+	(1)	515.0	22.0	18.0	23.5	626.7	6.7	–	–
	(2)	482.0	55.0	18.0	23.5	626.7	6.7	–	–
	(3)	427.0	110.0	18.0	23.5	626.7	6.7	–	–
Low Na^+	(1)	263.0	11.0	18.0	23.5	626.7	263.0	–	–
	(2)	105.0	11.0	18.0	23.5	626.7	421.0	–	–
	(3)	53.0	11.0	18.0	23.5	626.7	473.0	–	–
Na^+-free		–	11.0	18.0	23.5	626.7	526.0	–	–
Low Cl^-		526.0	11.0	18.0	23.5	100.7	6.7	526.0	–
Cl^--free*		526.0	11.0	18.0	23.5	6.7	6.7	573.0	23.5

For pH adjustment, less than 25 mM of Tris was additionally used in low Na^+ and Na^+-free solutions, and less than 2.0 mM in other solutions.

*This solution contained 6.7 mM Cl^- by the amount of Tris-HCl used for buffering.

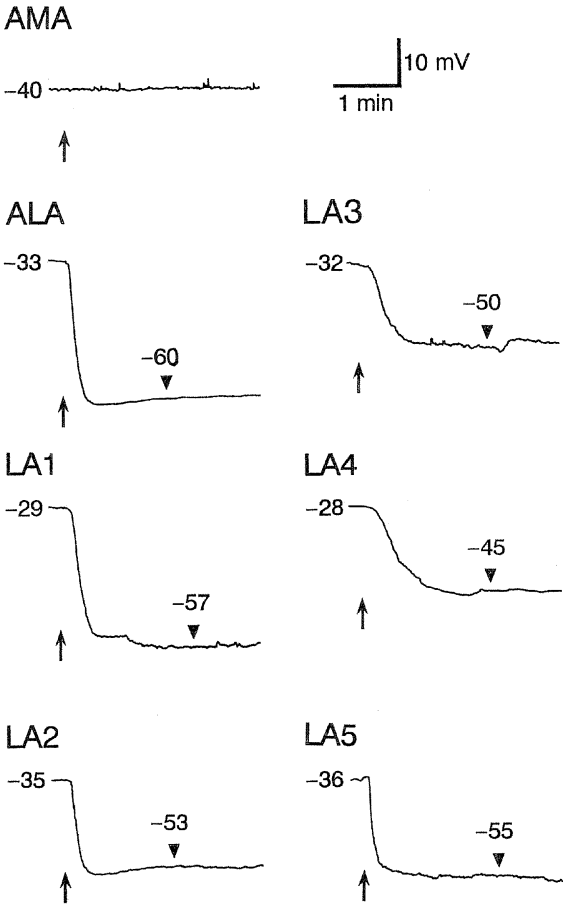


Fig. 1. Effects of ACh on the cardioarterial valves. The valve of the anterior median artery (AMA) was perfused with 10^{-4} M ACh beginning at the point indicated by an arrow. The valves of the anterior lateral artery (ALA) and the 1st to 5th lateral arteries (LA1-5) were perfused with 10^{-7} M ACh. Numerals at the beginning of traces and at arrowheads during each perfusion are membrane potentials (mV) in this and following figures.

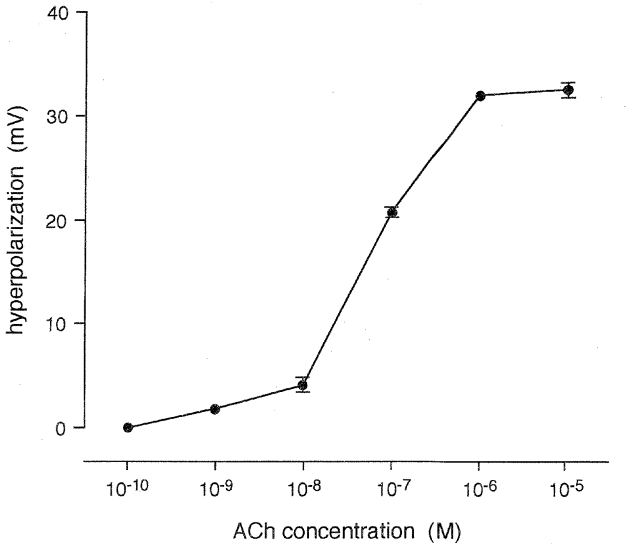


Fig. 2. The dose-response relationship between ACh and hyperpolarization of muscle cells of the cardioarterial valves. Plots and vertical bars show mean hyperpolarizing potential ± SE (N=5). SE bars were omitted when the values were smaller than the size of symbols.

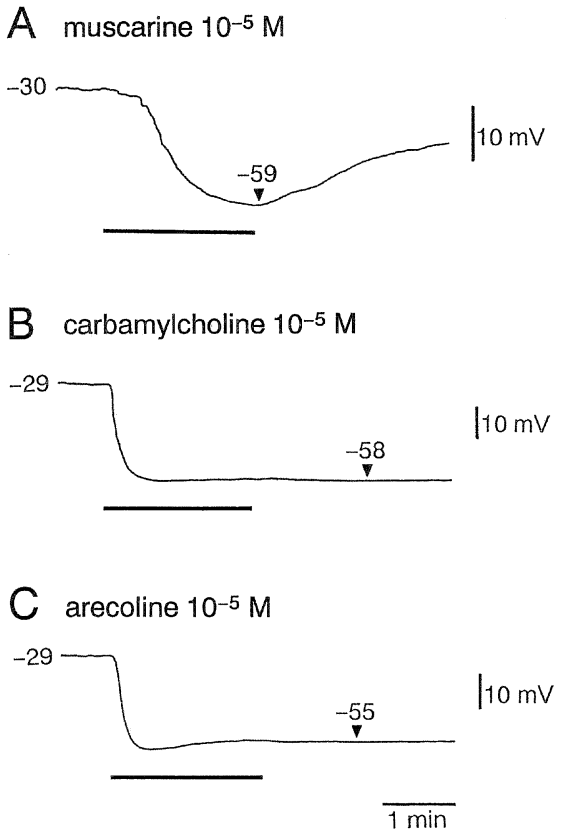


Fig. 3. Effects of muscarinic agonists on the LA valve. Underlining, periods of perfusion with agonists.

AMA did not respond at all to even high concentrations of ACh (up to 10^{-4} M).

Hyperpolarizing potentials in the valve of LA5 were induced in a dose-dependent manner by perfusion with ACh at 10^{-10} to 10^{-5} M (Fig. 2). The threshold concentration of ACh for the responses was between 10^{-10} M and 10^{-9} M.

Muscarine, carbamylcholine and arecoline, showed actions similar to ACh on valve muscle cells of LA5 (Fig. 3). Application of each agonist at 10^{-5} M to the bath induced 25-30 mV hyperpolarization of the valves, which was similar to the hyperpolarization induced by 10^{-7} to 10^{-6} M ACh (Fig. 2). Nicotine (10^{-4} M) caused depolarization by less than a few mV, and never caused hyperpolarization of the valve (not shown).

The valve of LA5 receives the 5th lateral cardiac nerve (LCN5) which consists of two valve inhibitory axons (Fujiwara-Tsukamoto *et al.*, 1992). Two discrete inhibitory junctional potentials (IJPs) of differing amplitudes were shown in valve muscle cells of LA5, in response to increasing intensity of single stimuli applied to LCN5 (Kihara *et al.*, 1985). These are smaller-sized single unitary IJPs and larger-sized compound IJPs (see Fig. 6A). Compound IJPs, generated by stimulation of the two LCN5 axons, were 16.8 ± 4.3 mV (mean ± SD, N=17) in am-

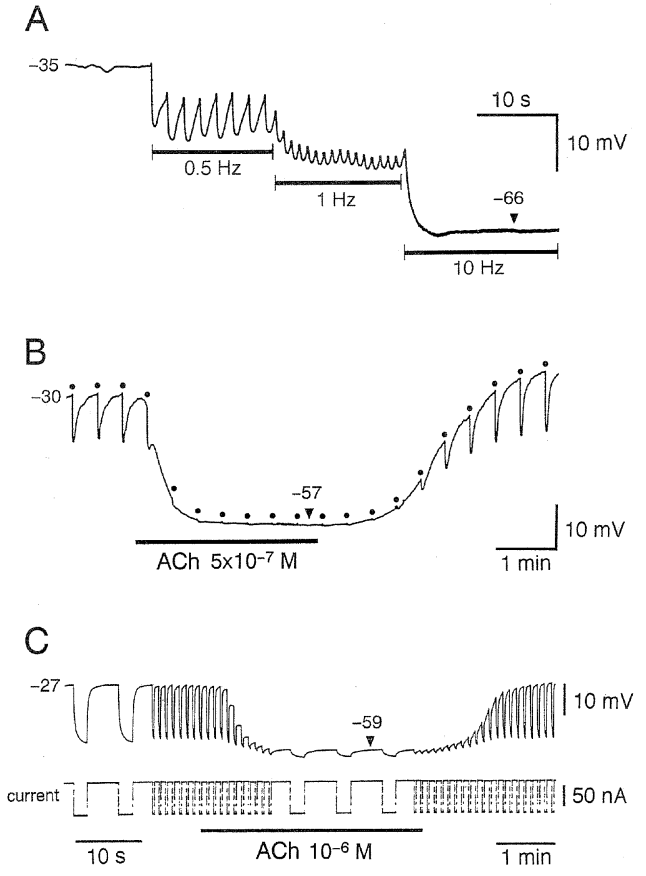


Fig. 4. (**A**) Trains of IJPs caused by LCN5 stimulation at various frequencies. Underlining indicates periods of nerve stimulation. (**B**) Effects of ACh on membrane potential of the valve during the occurrence of IJPs. Stimuli were applied at dots. (**C**) Effects of ACh on membrane conductance. Upper trace, intracellular recording; Lower trace, current monitor; Underlining, a period of perfusion with 10^{-6} M ACh. Faster sweep records are shown for the beginning of the trace and during the ACh treatment.

plitude. Figure 4A shows a train of compound IJPs caused by stimulation of LCN5 at various frequencies (0.5, 1 and 10 Hz). The resting membrane potential of the valve muscle cell (–35 mV) was hyperpolarized to –66 mV by repetitive stimuli at 10 Hz. When the specimen was perfused with 5×10^{-7} M ACh during trains of compound IJPs, membrane potential (–30 mV) was hyperpolarized to –57 mV, and the amplitude of the IJPs was reduced depending on the hyperpolarization (Fig. 4B). However, the hyperpolarizing IJPs were never inverted into depolarizing potentials. While recording the membrane potential, constant negative current pulses were applied through another microelectrode inserted into a muscle cell less than 200 μm apart from a recording electrode. ACh (10^{-6} M) reduced pulses in amplitude by about 78% at the most hyperpolarized level (–59 mV) (Fig. 4C).

Effects of cholinergic antagonists on arterial pressure

ACh markedly increased arterial pressure in LA5, in a dose-dependent manner (Fig. 5A). Figures 5B1 and B2 show effects of cholinergic blockers on the increased arterial pressure induced by stimulation of LCN5. Atropine (10^{-3} M) effectively blocked the response (Fig. 5B1). Methylxylocholine (10^{-4} M), which is known as a blocker for K^+-mediated ACh responses in gastropod neurons (reviewed by Gerschenfeld, 1973) and bivalve myocardium (Elliott, 1979, 1980), also blocked the response (Fig. 5B2).

Effects of cholinergic antagonists on IJPs and ACh-induced hyperpolarization

Figure 6 shows effects of cholinergic blockers on IJPs and ACh-induced hyperpolarizing potentials (ACh-potentials). Either unitary or compound IJPs could be elicited by means of changing stimulus intensity, and both were antagonized by atropine (5×10^{-4} M). In the same preparation, ACh-potentials were also effectively antagonized by atropine (5×10^{-5} M). Methylxylocholine antagonized both IJPs at 10^{-5} M and ACh-potentials at 10^{-6} M. The amplitude of IJPs was little affected by administration of *d*-tubocurarine (5×10^{-4} M), although it slightly shortened the time course of IJPs. However, the same concentration of *d*-tubocurarine effectively blocked ACh-potentials.

Effects of external K^+ and Na^+ concentrations on the resting membrane potential

Figure 7A shows membrane potentials of the valve muscle cells of LA5 in salines of various external K^+ concentrations ($[K^+]$). Successive lowering and increasing of $[K^+]$ from the concentration in ASW (11 mM) caused, respectively, hyperpolarizations and depolarizations. The slope of the change in membrane potential was 18.7 mV for a ten times $[K^+]$ at 18°C, which was far below the value (57.6 mV) predicted from the Nernst equation.

Effects of various external concentrations of Na^+ on resting membrane potential of the valve muscle cells are shown in Fig. 7B. With changes of Na^+ from 526 mM in ASW to lower concentrations, the membrane potential was hyperpolarized successively. The difference between mean resting membrane potentials in ASW (N=16) and in a Na^+-free solution (N=5) was 14.2 mV. Na^+ ions may contribute to keeping the resting membrane potential of valve muscle cells at the depolarized level.

Effects of external K^+ concentrations on IJPs and ACh responses

Effects of lowering external $[K^+]$ on IJPs and ACh-potentials are shown in Fig. 8A. When $[K^+]$ was reduced from 11 mM down to 1 mM, both IJPs and ACh-potentials increased in amplitude while the resting membrane potential was not altered. As $[K^+]$ was increased, amplitudes of IJPs and ACh-potentials decreased correspondingly (Fig. 8B). While the membrane potential was held at a resting level of –33 mV by current injection into a muscle cell through another microelectrode (see "holding" column of Fig. 8B), ACh-potentials were inverted to depolarizing potentials at 55 mM K^+, and at 110 mM K^+ the inverted potentials became larger in amplitude. On

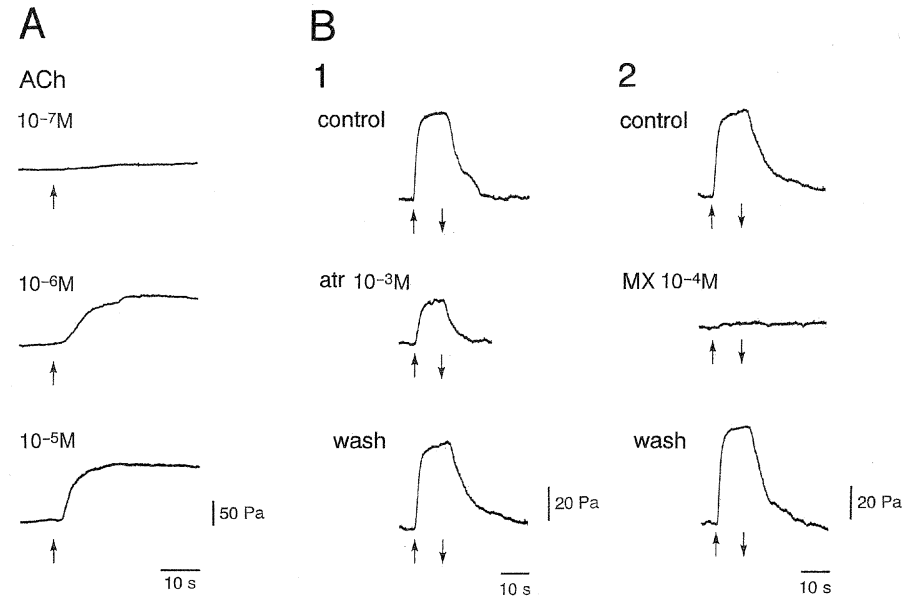


Fig. 5. (**A**) Effects of ACh on arterial pressure. Application of 10^{-7}–10^{-5} M ACh solutions (0.1 ml) to ASW supplying tube at arrows. (**B**) Effects of atropine (atr) (**1**) and methylxylocholine (MX) (**2**) on increases of the LA5 pressure induced by LCN5 stimulation. LCN5 was stimulated at 2 Hz between arrows.

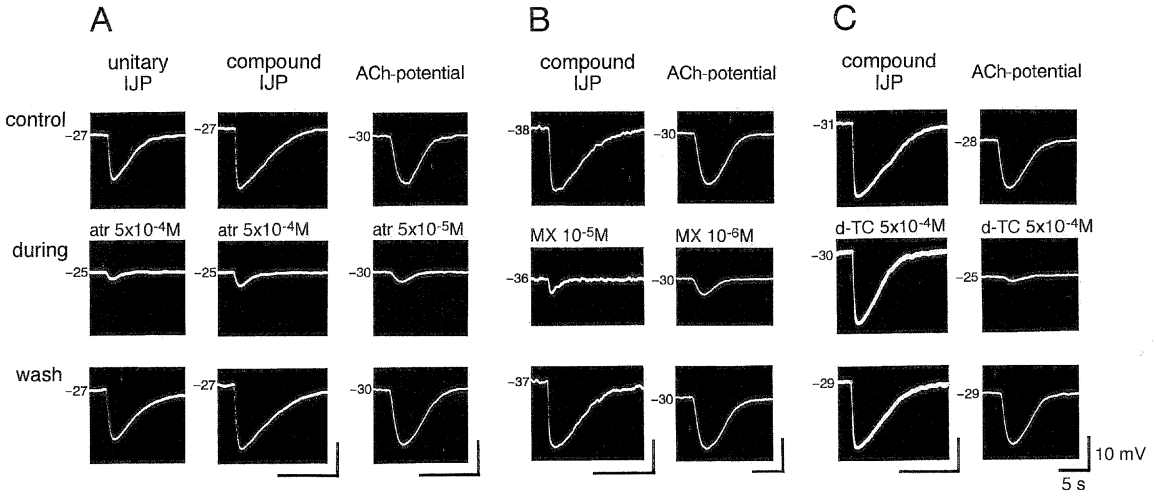


Fig. 6. Effects of cholinergic blockers on IJPs and ACh-potentials. (**A**) Atropine (atr), (**B**) methylxylocholine (MX), (**C**) *d*-tubocurarine (d-TC). In **A**, both unitary and compound IJPs are shown which were induced by stimuli of, respectively, low and high intensity applied to the dual-axon nerve LCN5. ACh-potentials were induced by iontophoretic application of ACh.

the other hand, IJPs disappeared in a 110 mM K^+ solution probably because of conduction blockage of the valve nerve (see Fig. 8B, third panel of IJP) even though the membrane potential was held around –25 mV (not shown). The inverted ACh-potentials were blocked by atropine (2×10^{-4} M) (not shown), as was the case with hyperpolarizing ACh-potentials in ASW (Fig. 6).

When Cl^--deficient solutions were applied, the membrane potential of valve muscle cells was depolarized or hyperpolarized. The depolarization and hyperpolarization reached about 10 mV and 20 mV, respectively. However, neither IJPs nor ACh-potentials were ever inverted into depolarizing potentials with the solutions.

DISCUSSION

Cholinergic inhibitory innervation

ACh is an excitatory neuromuscular transmitter in the stomatogastric system of the lobster (Marder, 1974, 1976; Lingle, 1980; Lingle and Auerbach, 1983). There is now no example of ACh as an neurotransmitter in neuromuscular junctions of crustacean skeletal muscles (reviewed by Atwood, 1982) since ACh, which was proposed as a excitatory neurotransmitter for crayfish abdominal flexor muscles (Futamachi, 1972), has

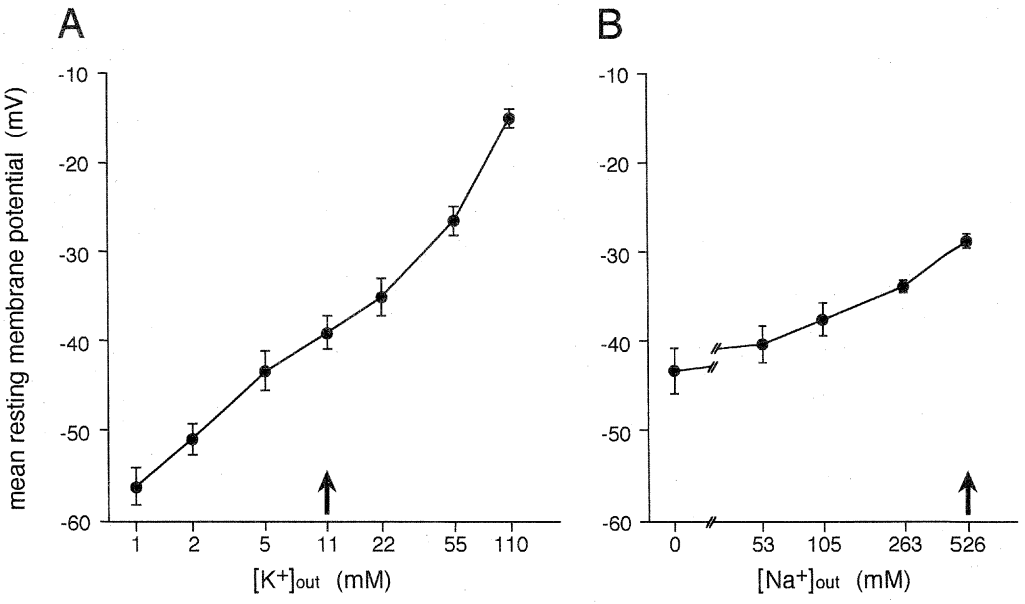
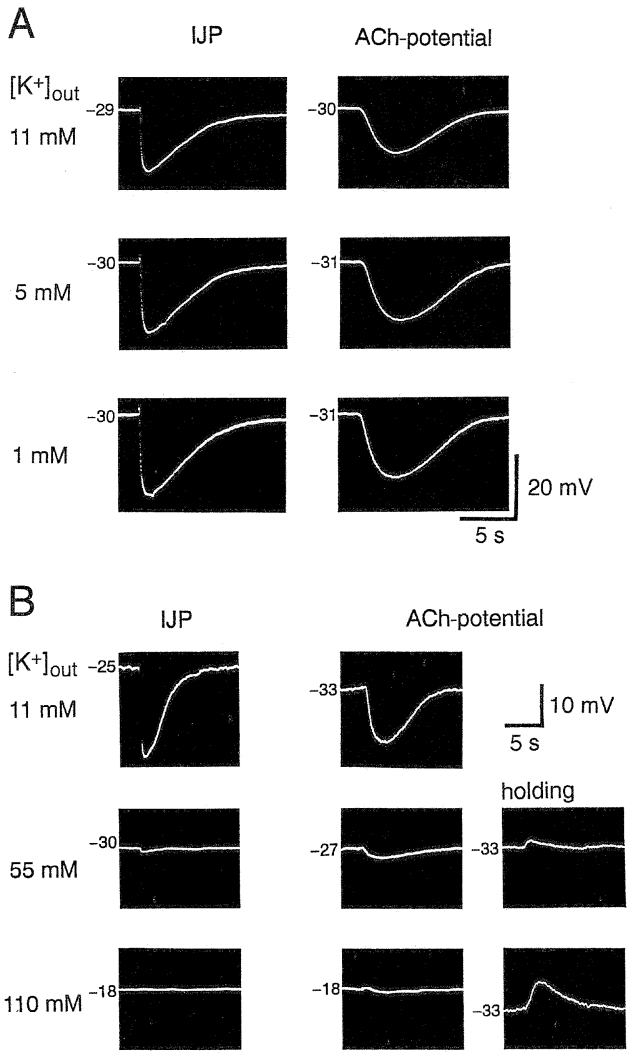


Fig. 7. Resting membrane potential of valve muscle of LA5 in various extracellular [K^+] (**A**) and [Na^+] (**B**). Plots and bars show mean value ± SE (N=5 to 14). Arrows show [K^+] and [Na^+] of normal ASW. [K^+] and [Na^+] are shown in log scale.



been replaced by glutamate (Kawagoe *et al.*, 1981). In the cardiovascular system of *Bathynomus doederleini*, ACh has been proposed as the transmitter of the extrinsic cardioacceleratory nerve innervating the myocardium together with the cardiac ganglion (Tanaka *et al.*, 1992). Cholinergic depolarizing responses in crustacean neurons have been involved in the mechanosensory system (Barker *et al.*, 1972; Miller *et al.*, 1992; Takashima *et al.*, 1996), stomatogastric system (reviewed by Marder, 1987), optic lobe (Pfeiffer and Glantz, 1989), cardiac ganglion (reviewed by Yazawa and Kuwasawa, 1992) and walking neural network (Cattaert *et al.*, 1995). Recently it was shown that the arterial valve muscle cells of *Homarus americanus* were depolarized by ACh (Wilkens and Davidson, 1995). Contrary to these examples, cholinergic hyperpolarizing responses in crustacean neurons were reported in the mechanosensory system (Barker *et al.*, 1972), stomatogastric system (Marder and Eisen, 1984) and optic lobe (Pfeiffer and Glantz, 1989). In the present specimens, the membrane potential of valve muscle cells was hyperpolarized by ACh in a dose-dependent manner (Kihara and Kuwasawa, 1985; Okada and Kuwasawa, 1993; and see Fig. 2). Muscarinic agonists mimicked the ACh effects. A muscarinic antagonist (atropine) and methylxylocholine, which is known as an ACh blocker in molluscs, blocked ACh-potentials and IJPs induced by inhibitory valve nerves. Administration of eserine (10^{-4} M) increased IJP amplitude (by 20.7 ± 9.2%, mean ± SD, N=6) and half-decay time (by 93.7 ± 58.7%, mean ± SD, N=6) (not shown).

Fig. 8. (**A**) Effects of low [K^+] salines on IJPs and ACh-potentials. (**B**) Effects of high [K^+] salines on IJPs and ACh-potentials. The resting membrane potential in the "holding" column was held at –33 mV by means of negative DC current injection.

In the cardiovascular system of some crustacean species, it is accepted that GABA is a common transmitter of the extrinsic cardioinhibitory nerve. Inhibitory effects of GABA on the heart were preferentially blocked by picrotoxin (Florey, 1957; Watanabe *et al*., 1968; Shimahara, 1969; Delaleu and Holley, 1976; Tanaka *et al*., 1992; reviewed by Yazawa and Kuwasawa, 1992; Yazawa and Kuwasawa, 1994). GABAergic inhibitory innervation was postulated for the cardioarterial valve (Kuramoto *et al*., 1992) and the arterial valves of the dorsal abdominal artery (Wilkens and Davidson, 1995) in the lobster *Homarus americanus*, because of similarity of hyperpolarizing effects of GABA and valve nerve stimulation on membrane potential of valve muscle cells. In the present material, however, even a high concentration of GABA (10^{-4} M) did not cause hyperpolarization of the membrane potential of the valve muscle cells at all. In the insect (Benson, 1988), mollusc (Yarowsky and Carpenter, 1978) and annelid (Schmidt and Calabrese, 1992) nervous system, as well as in the crustacean stomatogastric system (Marder and Paupardin-Tritsch, 1980), GABAergic blockers could antagonize ACh responses. In this study, picrotoxin (10^{-4} M) and bicuculline (2×10^{-4} M) did not reduce the amplitude of IJPs (not shown).

These results may present convincing evidence for the presence of cholinergic neural inhibition in the cardioarterial valves of LAs and ALAs. This cholinergic inhibition may be the first case described in studies of neuromuscular transmission of crustaceans.

Ionic mechanisms of IJPs and ACh-potentials

Inhibitory actions of ACh accompanying a decrease of K^+ conductance have been shown in motor neurons of the crayfish (Cattaert *et al*., 1994a, b). However, it was clearly shown in this study that, in *Bathynomus*, ACh increased the membrane conductance of valve muscle cells. In the stomatogastric system of the lobster, cholinergic pyloric neurons induced hyperpolarization of the lateral pyloric and pyloric constrictor neurons, accompanied by an increase of K^+ conductance (Marder and Eisen, 1984). ACh caused Cl^--dependent hyperpolarization in the neurons of the crayfish optic lobe (Pfeiffer and Glantz, 1989). Methylxylocholine blocks a K^+-mediated ACh response in the bivalve myocardium (Elliott, 1979, 1980). *d*-Tubocurarine blocks a Cl^--mediated ACh response in the myocardium (Kuwasawa and Yazawa, 1980; Kuwasawa *et al*., 1987; reviewed by Hill and Kuwasawa, 1990) and in the buccal muscles (Nagahama *et al*., 1993) of gastropods. In this study, IJPs were blocked by atropine and methylxylocholine, but not by *d*-tubocurarine (Fig. 6). On the other hand, ACh-potentials were blocked by *d*-tubocurarine as well as by both atropine and methylxylocholine. This pharmacological difference between IJPs and ACh-potentials may suggest that *d*-tubocurarine sensitive ACh receptors are distributed in the extrajunctional region. Amplitudes of both IJPs and ACh-potentials increased and decreased in low $[K^+]$ and high $[K^+]$ salines respectively (Fig. 8). Indeed, hyperpolarizing ACh-potentials were shown to be inverted into depolarization upon treatment with high $[K^+]$ salines. The inverted ACh-potentials were antagonized by atropine, as were the hyperpolarizing ACh responses. In foregut and opener muscles of the crayfish, Zufall *et al*. (1988) reported the existence of ACh-activated Cl^- channels. However, neither IJPs nor ACh-potentials inverted in Cl^--free saline in this study. We conclude that IJPs and ACh-potentials may be mediated mostly by an increase of K^+ conductance. It remains to be examined whether or not *d*-tubocurarine sensitive ACh receptors couple with K^+ and/or Cl^- channels.

Resting membrane potentials in the valves

The mean resting membrane potentials of valve muscle cells in all the valves (LAs, ALAs and AMA) were –36.5, –39.5 and –51.9 mV, respectively (Kihara *et al*., 1985). In the present study, the mean resting membrane potential in LA5 was –37.6 mV. Thus, muscle cells in the valves which receive inhibitory nerves have lower resting membrane potentials than those in the valves which receive excitatory nerves. This shows that the valves have more room for hyperpolarization of IJPs which causes relaxation of the valve muscle.

Stimulation of the LCNs at 10 Hz increased the amplitude of the pressure pulses in the LAs by 8.5 times (Fujiwara-Tsukamoto *et al*., 1992). In this study, stimulation of LCN5 at 10 Hz hyperpolarized membrane potential by more than 30 mV (see Fig. 4A). This may explain such a big increase of the pressure pulses. The following characteristics of valve muscle cells may be responsible for such a wide range in control of arterial haemolymph flow. 1) Resting membrane potentials of valve muscle cells are kept at a low level (about –38 mV). 2) The equilibrium potential for IJPs may be almost solely determined by K^+ ions which principally settle their equilibrium potential at a hyperpolarized level. 3) The individual IJPs have long time course of more than 5 sec (see Fig. 6A).

Changes of extracellular $[K^+]$ did not alter the resting membrane potential as predicted by the Nernst equation (Fig. 7A). Some mechanisms may contribute to maintain the low resting membrane potential. Lowering of external $[Na^+]$ hyperpolarized membrane potential (Fig. 7B). High Na^+ conductivity may contribute, at least in part, to the low resting membrane potential. Transient hyperpolarization was induced by replacing K^+-free saline by higher $[K^+]$ saline in valve muscle cells of LAs. This suggests the existence of an electrogenic Na^+ or Na^+-K^+ pump in valve muscle cells. However, ouabain (5×10^{-4} M) depolarized the resting membrane potential only by a few mV. As the valves of LAs do not receive constrictor (excitatory) nerves, the pump may function when muscle cells of the valves are depolarized by humoral substances. Octopamine, serotonin, norepinephrine and proctolin have been proposed as candidates for humoral substances causing contraction of the valves in *Bathynomus* (Tsukamoto and Kuwasawa, 1995).

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