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Inhibitory neural control of the myocardium in opisthobranch molluscs

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Key words. Opisthobranch gastropods; molluscan hearts; acetylcholine responses; inhibitory junctional potentials.

Introduction

Acetylcholine (ACh) has long been known as a cardioinhibitory agent in molluscs[4,8,11]. There was some evidence, however that ACh produced excitatory effects on the heart in a few species of bivalve[3]. In gastropods, the excitatory effect of ACh has been observed in *Strophocheilus*[7], and in *Helix*[18]. In the heart of *Helix*, the amplitude and tone are reduced although the frequency of action potentials may be increased[18]. Hill[5] demonstrated that ACh (2×10^{-8} to 2×10^{-3} M) depolarized the isolated ventricle of the opisthobranch *Dolabella auricularia*.

It has also been reported by Hill and Yantorno[6] that the ventricles of both *Dolabella auricularia* and *Aplysia dactylomela* were depolarized by ACh. Kuwasawa[13] showed that ACh may have different effects in different sites of the heart of *Dolabella*. The auriculo-ventricular valve (A–V valve) is usually hyperpolarized and the ventricle is depolarized.

Although the effects of ACh on the heart thus vary widely, even among gastropod species[8], it has been demonstrated in the *Dolabella* heart that neurally mediated cardiac inhibition itself may be cholinergic[13].

In *Aplysia californica*, the role of ACh as the transmitter of a heart inhibitor neuron has been established by biochemical and electrophysiological criteria[15]. On the other hand, clear evidence showing cholinergic inhibition has been obtained from the A–V valve of *Dolabella*. Muscle cells from which inhibitory junctional potentials (IJPs) are recorded are confined to the A–V valve[13]. Kuwasawa and Yazawa[14] showed that IJPs, induced by stimulation applied to the cardiac nerve, were clearly blocked by tubocurarine.

Materials and methods

The physiological significance of the responses of the myocardium to bath-applied ACh, and to ionophoretic application of ACh, were investigated with an intracellular recording technique. The purpose of the investigation was to elucidate what type of ACh response is involved in the physiological mechanisms of cardiac inhibition which are induced by regulatory nerves in opisthobranch molluscs.

Opisthobranch molluscs of the species *Dolabella auricularia*, belonging to the anaspidea, were collected on the seashore at Shimoda, and *Pleurobranchaea novaezealandiae*, belonging to the notaspidea was obtained from fishing boats in Yokohama. Both animals were stored separately at 15 °C in aquaria with natural seawater.

The experimental preparation was dissected in natural seawater from an animal injected with 5–15 ml isotonic $MgCl_2$ solution 5 min before the dissection. This preparation consisted of the heart alone or together with the cardiac nerve. In the case of *Pleurobranchaea*, the visceral (= abdominal) ganglion from which the cardiac nerve arises was included. The heart was cut longitudinally and pinned out flat in a small Sylgard-lined bath filled with artificial seawater

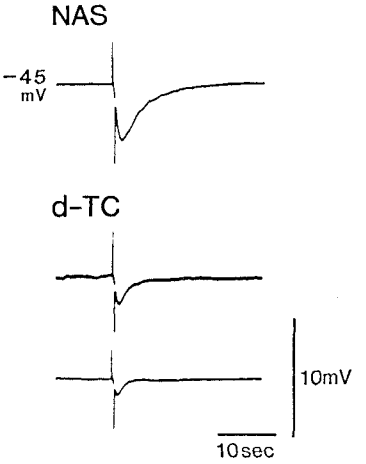

Figure 1. Effect of tubocurarine on the hyperpolarizing response to ACh of A–V valve muscle cells in *D. auricularia*. Top, control; middle and bottom, respectively, at 1 min and 3 min after the application of tubocurarine 2×10^{-5} M. In this and the following figures, numerals are membrane potentials at the beginning of each record.

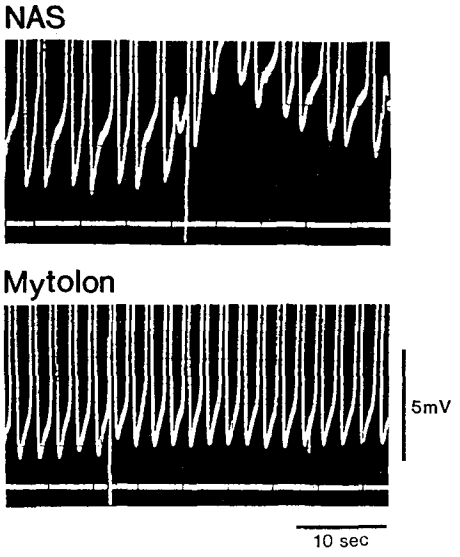

Figure 2. Effect of benzoquinonium on depolarizing responses to ACh of ventricular muscle cells of *D. auricularia*. Upper, control in normal ASW (NAS); lower, in NAS containing mytolon (benzoquinonium) (10^{-5} M). The time course of the changes in membrane potential, in response to ACh ionophoresis, can be recognized from the level of the maximum hyperpolarizing potentials in each cardiac cycle. Action potentials are truncated. Upper and lower traces in each figure show, respectively, records of membrane potential and ACh pulses. Calibration: 300 ms, 300 nA.

(ASW). Preparations were perfused with aerated ASW using gravity feed and suction removal, allowing penetration of muscle cells from the inside of the heart. The cut end of the cardiac nerve was sucked into a glass capillary electrode for electrical stimulation. In some cases major parts of the ventricle and the auricle were removed in making preparations, in order to avoid strong beats, which make long intracellular recording with microelectrodes difficult.

Na propionate or Na methansulfonate was substituted for NaCl to prepare Cl^--deficient ASW. The composition of the ASW used was the same as previously reported[14], except that HEPES instead of Tris was used as a buffer.

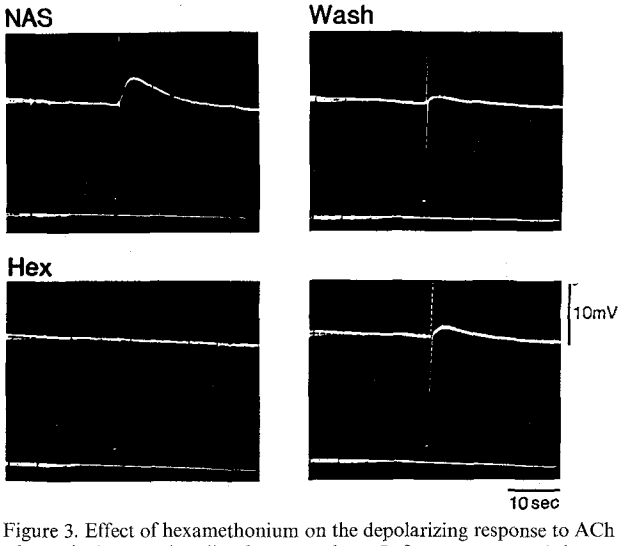

Figure 3. Effect of hexamethonium on the depolarizing response to ACh of ventricular muscle cells of *D. auricularia*. Left: upper, control; lower, hexamethonium (10^{-4} M). Right: upper and lower, wash with normal ASW at, respectively, 5 and 10 min after the cessation of hexamethonium application. Upper and lower traces in each figure show, respectively, records of membrane potential and ACh pulses. Calibration: 300 ms, 100 nA.

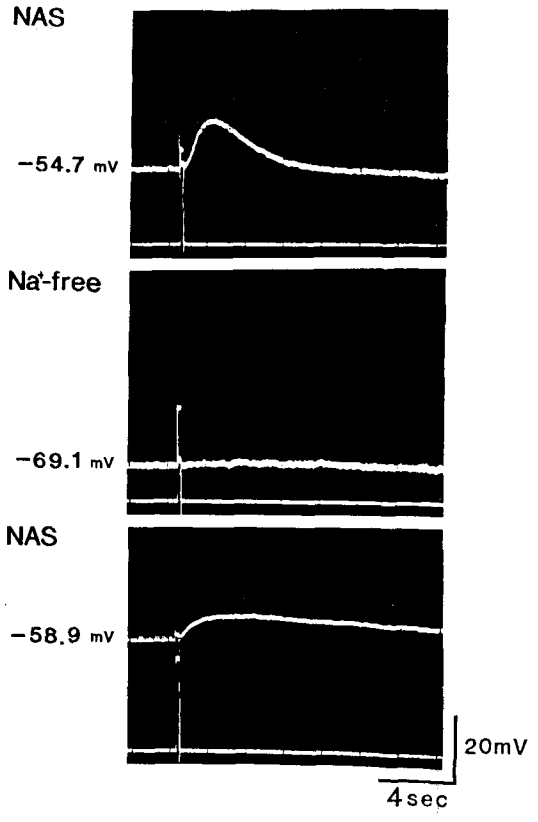

Figure 4. Effect of Na^+-free ASW on the depolarizing response to ACh of ventricular muscle cells in *D. auricularia*. Top, control; middle, Na^+-free ASW; bottom, during a wash with normal ASW. Upper traces and lower traces in each figure show, respectively, records of potential and ACh pulses. Calibration: 200 ms, 300 nA.

Electrical recording

Glass microelectrodes filled with 3 M KCl, which had tip resistances of 25–50 Megohms, were used for intracellular recording, since no differences in ACh responses or in effects of ion substitutions were detectable with electrodes filled with different kinds of solutions[2]. An Ag-AgCl reference electrode communicated with the bath via a 3 M KCl reservoir and an agar bridge. The reference electrode was positioned at the site where perfusate was removed by suction. The agar bridge was made with a glass tube of 1 mm inside diameter, filled with 2% agar in 3 M KCl. Membrane potentials were recorded using an oscilloscope (Tektronix 5110) and a pen-writing oscillograph (Nihon Kohden WI-641 G).

Ionophoresis

Ionophoretic micropipettes were filled with 1 M ACh. Resistance of the electrodes ranged from 10 to 40 megohm. Current pulses of 100–300 ms and 100–300 nA were delivered using a ionophoresis device (Nihon Kohden MEZ-8201). Retaining currents of 2–5 nA were usually used. The ACh pipette was positioned as close to the recording site as possible.

Chemicals

The following antagonists were used: d-tubocurarine chloride (Kanto Chemical), hexamethonium chloride (Wako Pure Chemical), benzoquinonium chloride (also called mytolon, a gift from Sterling-Winthrop Research Institute, Rensselaer, New York) and methylxylocholine chloride (also called β-methyl TM 10, a gift from Smith, Kline & French Laboratories, Philadelphia, Pennsylvania).

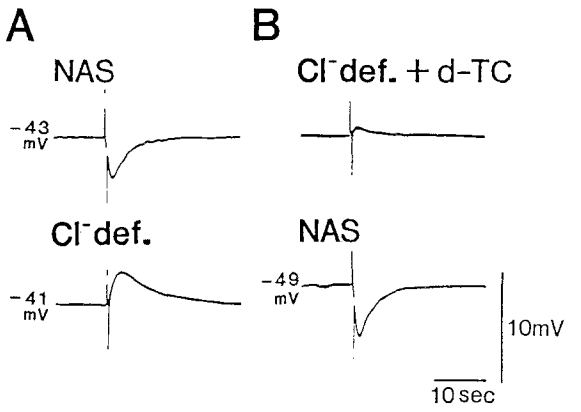

Figure 5. Effects of Cl^--deficient ASW and tubocurarine on the hyperpolarizing response to ACh of A–V valve muscle cells in *D. auricularia*. *A* Upper, control; lower, inverted ACh response in Cl^--deficient ASW. *B* Upper, tubocurarine (2×10^{-5} M) in Cl^--deficient ASW; lower, wash with normal ASW.

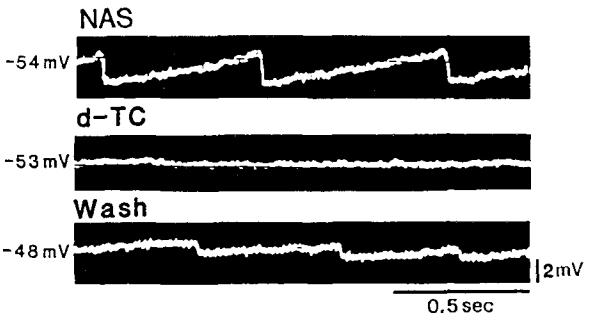

Figure 7. Effect of tubocurarine on unitary IJPs recorded from the auricle of *P. novaezealandiae*. Individual IJPs were induced repetitively by one stimulus pulse applied to the branchial nerve. Top, control; middle, tubocurarine (10^{-5} M); bottom, wash with normal ASW.

Results

Pharmacological properties of ACh responses

Two different ACh responses, depolarization and hyperpolarization, recorded respectively from the ventricle and the A–V valve, both resulted from a decrease in membrane resistance[13]. The ACh responses were differentially sensitive to the cholinergic antagonists: mytolon, tubocurarine, hexamethonium and methylxylocholine. Mytolon was chosen since it is effective in blocking cholinergic effects on bivalve hearts[1, 3a,b, 16] and on the heart of the gastropod, *Lymnaea*[17]. The other drugs were chosen because they have been found to be effective in distinguishing the three types of ACh response in *Aplysia* neurons[9, 10, 19] and in bivalve hearts[2].

Tubocurarine clearly antagonized the hyperpolarization of the A–V valve caused by ACh pulses of 100 ms and 200 nA (fig. 1). Mytolon blocked the depolarizing response of the ventricle (fig. 2) and the hyperpolarizing response of the A–V valve to ACh. Although this substance has been used as an antagonist of ACh-induced inhibition of bivalve hearts, similar non-selective action of mytolon against depolarizing and hyperpolarizing responses to ACh was found in bivalve hearts[2]. Methylxylocholine did not block either depolarization of the ventricle or hyperpolarization of the A–V valve of *D. auricularia* caused by ACh pulses. Hexamethonium blocked ACh-induced depolarizing responses in the ventricle (fig. 3). It has been reported that hexamethonium blocked rapid Na^+ mediated ACh responses in muscle cells of bivalve heart[2].

Ionic mechanisms of the depolarizing and hyperpolarizing responses

In Na^+-free artificial seawater (ASW), bath-applied ACh did not affect polarization of the ventricle[13]. This finding was confirmed with the technique of ionophoretic application of ACh. In figure 4, the top trace shows depolarization induced by an ACh pulse in normal ASW. The response was abolished by perfusion with Na^+-free ASW (middle trace), and the depolarizing response recovered in normal ASW (bottom trace). In normal ASW, a hyperpolarizing response to ionophoretic ACh application was obtained only from the A–V valve. The hyperpolarizing response to an ACh pulse (100 ms and 200 nA) (fig. 5A, upper trace) was inverted into a depolarizing response in Cl^--deficient ASW (fig. 5A, lower trace). The inverted response to ACh in Cl^--deficient ASW was antagonized by tubocurarine (fig. 5 B), as was the normal response.

Pharmacological properties of inhibitory junctional potentials

Individual inhibitory junctional potentials (IJPs), which gave a one-to-one response to stimulus pulses applied to cardiac nerves, were recorded from the A–V valve in *Dolabella*[12–14]. The IJPs were preferentially blocked by tubocurarine[14]. In this study, we found that mytolon also blocked IJPs in the *Dolabella* heart. Hexamethonium and methylxylocholine did not block the IJPs. These pharmacological properties of IJPs are analogous to the properties of hyperpolarizing responses to ionophoretic application of ACh.

We employed another opisthobranch gastropod, *Pleurobranchaea novaezealandiae*, instead of *Dolabella*, to investigate pharmacological properties of the cardiac inhibition which is induced by cardio-regulatory nerves. Figure 6 shows

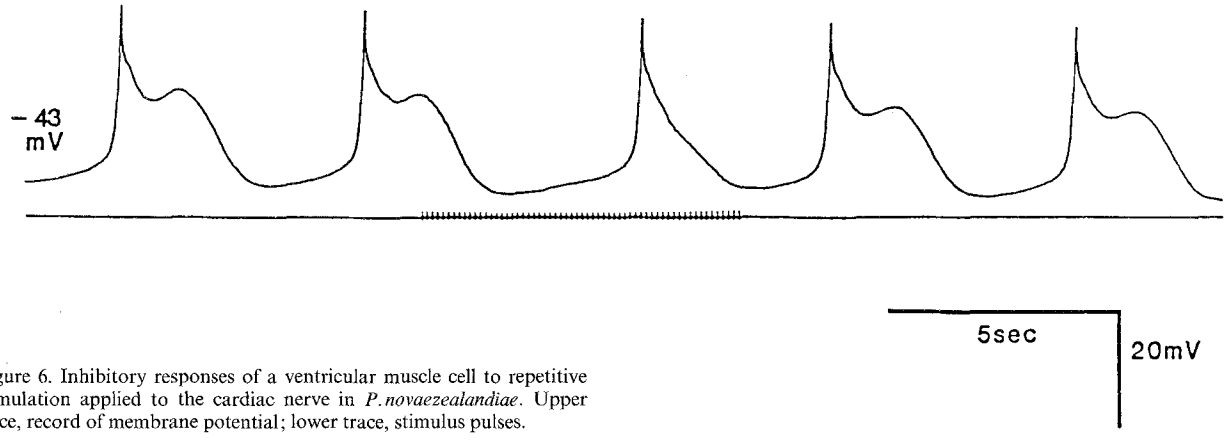

Figure 6. Inhibitory responses of a ventricular muscle cell to repetitive stimulation applied to the cardiac nerve in *P. novaezealandiae*. Upper trace, record of membrane potential; lower trace, stimulus pulses.

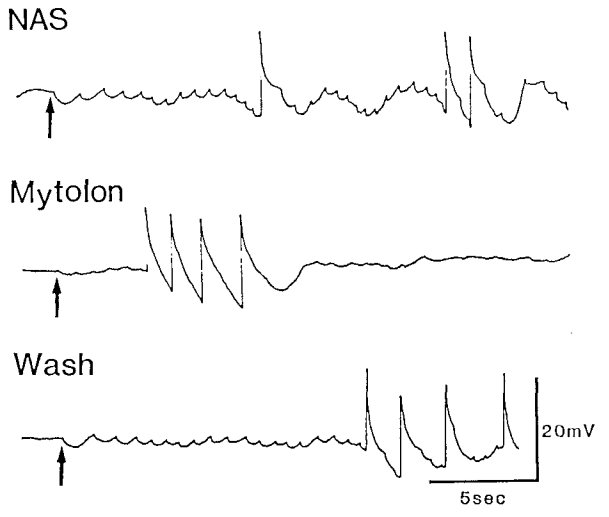

Figure 8. Effect of benzoquinonium on IJPs recorded from the auricle of *P. novaezealandiae*. Top, control; middle, mytolon (benzoquinonium) (10^{-5} M); bottom, wash with normal ASW. A train of IJPs were induced by single stimulus pulses applied to the branchial nerve at arrows. Resting membrane potential in three records, − 52 mV.

the inhibition recorded from the ventricle during a period of repetitive stimulation applied to the cardiac nerve. Hyperpolarization during the stimulation was accompanied by a negative chronotropic effect which was observed as an elongation of the interval between action potentials and a shortening of the plateau phase of action potentials during the stimulation. Individual IJPs could be discerned in the hyperpolarization of the auricle and A–V valve, but only rarely and obscurely from the ventricle. The IJPs were blocked by perfusion with tubocurarine (10^{-5} M) (fig. 7). Mytolon also blocked IJPs (fig. 8). Hexamethonium and methylxylocholine did not antagonize IJPs. These pharmacological features of IJPs in the *Pleurobranchaea* heart are the same as those of IJPs in the *Dolabella* heart.

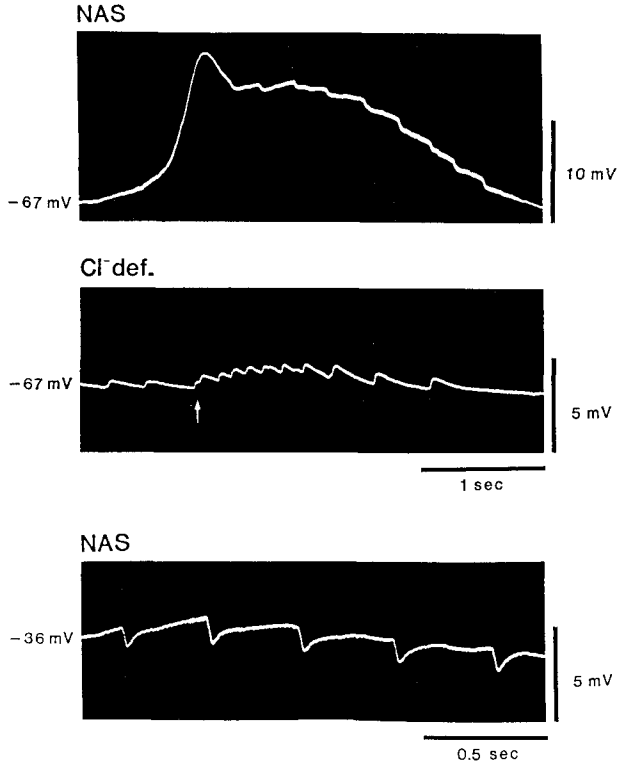

Figure 9. Effect of Cl^--deficient ASW on IJPs recorded from the auricle of *P. novaezealandiae*. Top, control; middle, Cl^--deficient ASW; bottom, wash with normal ASW. IJPs in the top trace were induced repetitively in response to one stimulus pulse applied to the branchial nerve at a time before the beginning of record. IJPs which occur before the arrow in the middle trace were induced by a previously applied single stimulus pulse to the nerve, and IJPs after the arrow were induced by another single pulse to the nerve.

Ionic mechanisms of IJPs

We observed not only ACh-induced hyperpolarizing responses in the A–V valve of *Dolabella*, but also hyperpolarizing normal IJPs which were inverted into depolarizing IJPs when the A–V valve was perfused with Cl^--deficient ASW[14]. Similar experiments were performed in the *Pleurobranchaea* heart. The preparation used in the experiments was isolated together with the nervous system, including nerves to the heart and the visceral ganglion from which the nerves arise, so that one of the preganglionic nerves (the branchial nerve) could be used to induce IJPs in the heart. IJPs shown in figure 9 were obtained from the experiments. The top trace shows normal IJPs evoked in the auricle by repetitive stimuli applied to the branchial nerve, during perfusion of normal ASW. The middle trace shows inverted IJPs during perfusion of Cl^--deficient ASW. In the middle trace, both spontaneous IJPs (before the arrow) and induced IJPs (after the arrow) were inverted into depolarizing potentials by perfusion with Cl^--deficient ASW. The bottom trace was obtained during a wash with normal ASW, showing recovery of IJPs. One stimulus pulse had been applied to the nerve before the beginning of the record.

Conclusions

The results obtained from *Dolabella auricularia* and *Pleurobranchaea novaezealandiae* appear the same with regard to pharmacological and ionic properties of IJPs. Furthermore, pharmacological and ionic properties of the hyperpolarizing ACh responses in the heart of *Dolabella* also seem to be the same as those of IJPs in the hearts of *Dolabella* and *Pleurobranchaea*.

We may summarize these results as shown in the table. As Na^+-dependence of IJPs could not be investigated, since Na^+-dependent solutions produced prejunctional effects on inhibitory nerves, the minus signs within parentheses represent a speculation based on indirect evidence that IJPs lack Na^+-dependence. The indirect evidence is the observation that hexamethonium has no effect on IJPs or on the hyperpolarizing ACh response. Hexamethonium has been known to block Na^+-mediated depolarizing ACh responses in gastropod neurons[10,19]. The present results show that the depolarizing ACh response was abolished with Na^+-free ASW and that the response was blocked by hexamethonium. This is congruent with the action of hexamethonium on the ACh response in bivalve hearts[2].

Tubocurarine has been shown to be an effective blocker of Cl^--mediated ACh responses even though the response appears in the depolarizing direction in bivalve hearts[2]. Tubocurarine clearly antagonized the hyperpolarizing ACh response in the A–V valve of *Dolabella*. IJPs in the hearts of both *Dolabella* and *Pleurobranchaea* were strongly antagonized by tubocurarine.

Since IJPs in the heart could be expected at least to play some role, in the primary process of cardiac inhibition mediated by neuro-muscular junctions in the heart, the cardiac inhibition of the heart of opisthobranchs may involve a Cl-mediated ACh response. The Cl-mediated mechanism of ACh responses may be a major mechanism, because inverted depolarizing IJPs in Cl^--deficient ASW were almost totally abolished by tubocurarine.

Ionic and pharmacological characteristics of the hyperpolarizing ACh responses and IJPs. *A* + or − indicates presence or absence of a blocking effect. *B* + or − indicates presence or absence of ionic dependence. ( ), indirect evidence

	ACh H-response	IJP
A) Mytolon	+	+
Tubocurarine	+	+
Methylxyolocholine	−	−
Hexamethonium	−	−
B) Cl^--dependence	+	+
Na^+-dependence	−	(−)

The methylxylocholine-sensitive K^+-mediated response to ionophoretic ACh application, which is found in bivalve myocardium[2], was not found in the present materials. The physiological role of the Na^+-mediated depolarizing ACh response is not yet known. Further investigation of neuronal and neurohumoral control of the myocardium may elucidate these subjects further.

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Cardiac and circulatory control in decapod Crustacea with comparisons to molluscs

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Summary. In this review I will attempt to identify the circulatory requirements a decapod is likely to encounter and how the heart is controlled to meet these demands. The decapod heart has been designed as an autonomous system endowed with an intrinsic autorhythmic pacemaker ganglion. Muscle fibers are multiply-innervated and capable of producing regenerative action potentials. This vitally important organ has been designed to be nearly fail-safe. Stroke volume is more important than heart rate in determining cardiac output. Stretch sensitivity of the cardiac ganglion and of the myocardium as well as extrinsic nervous and hormonal modulation of the heart can all contribute to changes in stroke volume. It may be advantageous to an animal to switch the circulation between various vascular beds to meet changing perfusion demands. Neuronal and hormonal mechanisms have been identified which exert differential control of the cardioarterial valves, but it is not known whether switching does occur and if so whether these valves participate in the process. Changes in peripheral resistance can also redirect circulatory flow. The circulatory and ventilatory systems demonstrate coordinated rate changes which suggest that the heart is responding to meet changing ventilatory performance requirements. This coupling is controlled both by the hydrostatic pressure pulses generated within the branchial chambers and by common higher level nervous inputs. Comparisons of the cardiovascular systems of crustaceans and molluscs, based on the papers presented at this symposium, are high-lighted.
Key words. Decapod Crustacea; cardiac control; cardiac output.

Introduction

The isolated heart, the cardiac ganglion (CG) and the regulatory nerves to the heart have received considerable research attention where the primary focus has been to understand the physiology of the particular tissue in question. Heart rate