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RESEARCH ARTICLE

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# Control of locomotion in marine mollusc Clione limacina X. Effects of acetylcholine antagonists

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**Abstract** The swimming central pattern generator (CPG) of the pteropod mollusc *Clione limacina* is located in the pedal ganglia. It consists of three groups of interneurons (7, 8, and 12) which generate the rhythmical activity and determine the temporal pattern of the motor output, that is, phasic relations between different groups of motor neurons supplying dorsal (group 1 and 3 motor neurons) and ventral (group 2 and 4 motor neurons) muscles of the wings. In this work peripheral and central effects of acetylcholine (ACh) antagonists on the swimming control in *C. limacina* has been studied. The ACh antagonist atropine blocked transmission from the wing nerves to wing muscles, while gallamine triethiodide (Flaxedil), *d*-tubocurarine, and α-bungarotoxin did not affect the neuromuscular transmission. In the pedal ganglia, the ACh antagonists atropine and gallamine triethiodide blocked inhibitory postsynaptic potentials (IPSPs) produced by group 8 interneurons onto group 7 interneurons and motor neurons of groups 1 and 3. *d*-Tubocurarine and α-bungarotoxin did not affect IPSPs produced by group 8 interneurons. Although atropine and gallamine triethiodide blocked IPSPs produced by group 8 interneurons in antagonistic neurons, these drugs did not influence excitatory postsynaptic potentials (EPSPs) produced by group 8 interneurons onto group 12 interneurons. The main pattern of the swimming rhythm with an alternation of two phases of the swimming cycle persisted after elimination of inhibitory connections from group 8 interneurons to antagonistic neurons by the ACh antagonists. This suggests that there are redundant mechanisms in the system controlling *C. limacina's* swimming. This redundancy ensures reliable operation of the system and contributes to its flexibility.

**Key words** Locomotion · Central pattern generator · Acetylcholine antagonists · Pteropod mollusc

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## Introduction

The pteropod mollusc *Clione limacina* (class: Gastropoda; subclass: Opisthobranchaea) lives in the cold waters of northern oceans. This mollusc swims by continuous rhythmical movements (1–2 Hz) of two wings (Arshavsky et al. 1985a; Lalli and Gilmer 1989; Litvinova and Orlovsky 1985; Satterlie et al. 1985; Wagner 1885). Each locomotor cycle consists of two phases, a dorsal wing flexion (D-phase) and a ventral wing flexion (V-phase) produced by alternating contractions of antagonistic muscles.

In the preceding articles of this series the neural mechanisms controlling *C. limacina's* swimming were described (Arshavsky et al. 1985a–e, 1986, 1989). The swimming rhythm is generated by a central mechanism (the central pattern generator, CPG) which is mainly located in the pedal ganglia. Figure 1A illustrates the organization of the neural network controlling swimming in *C. limacina*. The network can be divided into two half-centers, consisting of neurons active in D- and V-phases of the swimming cycle (D-phase and V-phase half-centers). According to their morphology, neurons involved in the control of swimming are divided into motor neurons with axons projecting in the wing nerves and interneurons. Three groups of interneurons (groups 7, 8, and 12) form the swimming CPG. Group 7 and 8 interneurons, active in D- and V-phases of the cycle, respectively, play a crucial role in the rhythm generation. There are about ten cells of each group per ganglion. Axons of the interneurons project into the contralateral ganglion. The interneurons generate action potentials which have a long duration (about 100 ms); the interneurons of two groups inhibit each other. All interneurons of a given

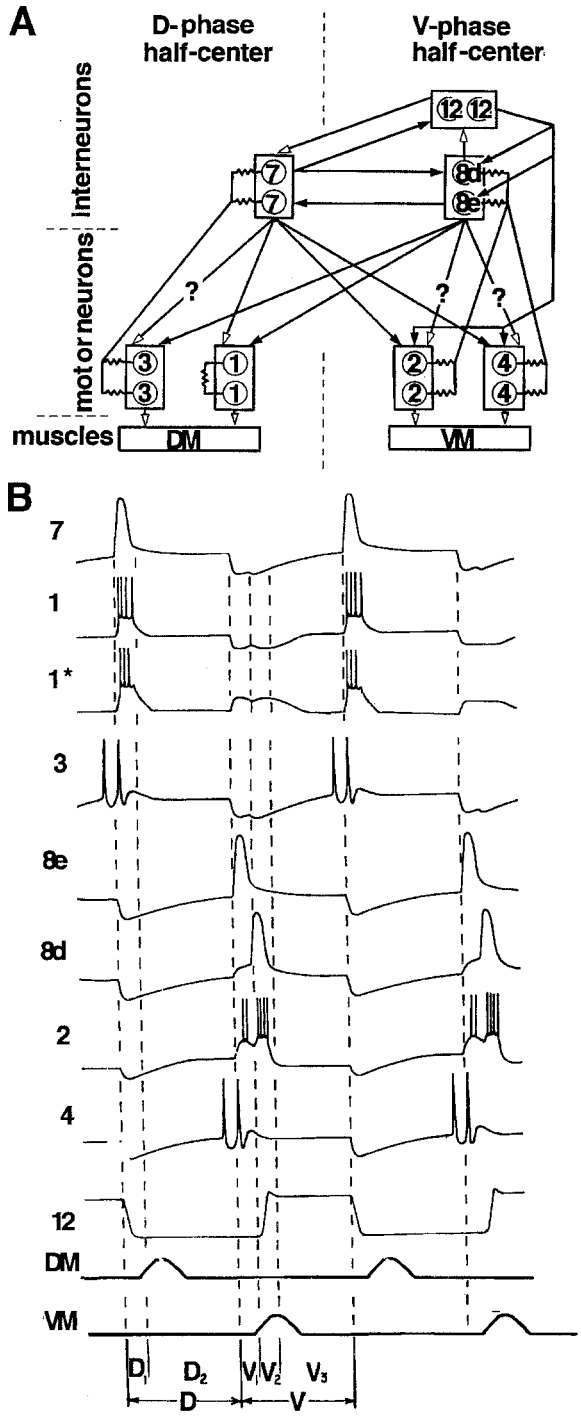


**Fig. 1** **A** Neuronal network controlling rhythmical wing movements in *Clione limacina*. Electrical connections are shown by *resistor symbols*, excitatory chemical connections by *white arrows*, and inhibitory connections by *black arrows*. *Question marks* indicate putative connections which were not proved. *DM* and *VM*, dorsal and ventral wing muscles. **B** Phases of the activity of different groups of neurons in the swimming cycle. *1* and *1** illustrate the activity of group 1 motor neurons with normal and inverted inhibitory postsynaptic potentials. Mechanograms of dorsal and ventral wing muscles are also shown. See text for details

group from both ganglia are electrically interconnected. As a result, group 7 interneurons are excited simultaneously. On the other hand, group 8 interneurons are subdivided into two subgroups, the "early" (8e subgroup) and "delayed" (8d subgroup), which are excited with some lag due to a higher threshold of the interneurons 8d. The third group of interneurons, group 12, are located in the pleural ganglia. There are two group 12 interneurons, one neuron per ganglion. Their axons enter the pedal ganglia and form two nets of neuropilar processes in the lateral areas of the ganglia. Group 12 interneurons generate "plateau" potentials of a constant amplitude. In other words, these cells have two stable levels of membrane potential, the "lower" one (the resting potential) and the "upper" one, when the neuron is depolarized (Arshavsky et al. 1985d, 1989). Group 12 interneurons receive an excitatory input from interneurons of the 8d subgroup and inhibitory input from group 7 interneurons. In their turn, group 12 neurons inhibit neurons of the V-phase (group 8 interneurons and motor neurons) and exert some excitatory action on group 7 interneurons. Group 12 interneurons are not connected to each other.

Group 7 and 8 interneurons control wing muscle motor neurons. They excite motor neurons of the same half-center and inhibit antagonistic motor neurons. Unlike interneurons, motor neurons generate ordinary spikes (1–5 ms). There are four main groups of motor neurons. Groups 1 (about 15 cells in each ganglion) and 3 (about five cells) supply the dorsal wing muscle, while groups 2 (about ten cells) and 4 (about five cells) supply the ventral wing muscle. Group 1 motor neurons include the large, visually identifiable 1A motor neuron, while group 2 includes the large, visually identifiable 2A motor neuron. Group 1 motor neurons have no electrical connections with synergetic group 7 interneurons. Therefore, excitatory action of group 7 interneurons on group 1 motor neurons is realized through chemical synapses. Motor neurons of all other groups have electrical connections with interneurons of the same half-center. Therefore, excitatory action of group 7 interneurons on group 3 motor neurons and of group 8 interneurons on group 2 and 4 motor neurons is realized through electrical synapses. Some indirect results suggest that there also exist chemical excitatory connections from group 7 interneurons to group 3 motor neurons and from group 8 interneurons to motor neurons of groups 2 and 4. However this subject needs further study.

The anatomical structure and innervation of *C. limacina's* wing muscles were studied by Satterlie (1991, 1993). It was shown that the wing muscles consist of fast-twitch and slow-twitch fibers. The large motor neurons (1A and 2A) innervate both types of muscle fibers, while small motor neurons innervate only slow-twitch fibers.

Figure 1B shows schematically the temporal pattern of the activity of different groups of neurons during the swimming cycle. As was mentioned above, the cycle consists of the two main phases, D and V. The D-phase starts with the synchronous excitation of group 7 interneurons (the $D_1$-phase). Group 7 interneurons produce excitatory

postsynaptic potentials (EPSPs) in motor neurons of groups 1 and 3, and inhibitory postsynaptic potentials (IPSPs) in group 2 and 4 motor neurons as well as in group 8 and 12 interneurons. As a result, group 12 interneurons pass to the lower state in this phase. The duration of the $D_1$-phase is determined by the duration of action potentials generated by group 7 interneurons. This phase is followed by the $D_2$-phase when no new postsynaptic potentials appear. The V-phase starts with the synchronous excitation of subgroup 8e interneurons (the $V_1$-phase). The excitation of these neurons is determined both by their capacity for endogenous rhythmic activity (Arshavsky et al. 1986) and by the postinhibitory rebound after termination of the IPSP arising in the D-phase (Arshavsky et al. 1985c; Satterlie 1985, 1989). Group 8e interneurons produce, due to electrical connections, depolarization of subgroup 8d interneurons, which fire with some delay after excitation of subgroup 8e interneurons (the $V_2$-phase). The excitation of subgroup 8e and 8d interneurons produces two-component EPSPs in group 2 and 4 motor neurons and two-component IPSPs in group 7 interneurons and group 1 and 3 motor neurons (since an equilibrium potential for IPSPs in group 1 motor neurons is close to their membrane potential, IPSPs recorded from these motor neurons were frequently reversed; cf. 1 and 1* in Fig. 1B). In addition, subgroup 8d interneurons produce EPSPs in group 12 interneurons, passing them into the upper (depolarized) state. The $V_2$-phase is followed by the $V_3$-phase in which group 12 interneurons are the only source of postsynaptic potentials. They mainly exert an inhibitory action on neurons active in the V-phase. The swimming cycle is finished with new excitation of group 7 interneurons. There are three reasons for their excitation: the capability of the neurons for endogenous rhythmical activity, the postinhibitory rebound, and the excitatory input from group 12 interneurons.

In addition to postsynaptic potentials, motor neurons generate spike discharges in the proper phases of the cycle. In group 3 and 4 motor neurons spike discharges may start earlier than EPSPs as a result of a postinhibitory rebound. In some cases, motor neurons do not generate spike discharges during the swimming rhythm. This was especially typical for the large 1A and 2A motor neurons (see also Satterlie 1993).

Neurotransmitters used by motor and interneurons of the network controlling swimming in *C. limacina* have not been identified. As the first step in this direction, we have studied the effects of acetylcholine (ACh) antagonists on the swimming control in *C. limacina*. We show that: (1) transmission from motor neurons to wing muscles is blocked by atropine; and (2) an inhibitory action of group 8 interneurons onto antagonistic interneurons and motor neurons is blocked by atropine and gallamine triethiodide. These results suggest that cholinergic mechanisms play an important role in the control of *C. limacina's* swimming. Effects of blockage of inhibitory inputs from the group 8 interneurons to antagonistic neurons on an operation of the swimming CPG have also been studied.

## Materials and methods

Experiments were carried out at the White Sea Marine Biological Station, *Kartesh*. Specimens were obtained locally and kept in a seawater aquarium at 5°C. Intact adult molluscs (3–5 cm long) were used to study the behavioral effects of ACh antagonists. Among ACh antagonists, atropine, gallamine triethiodide, *d*-tubocurarine, and α-bungarotoxin (all from Sigma) were used. They were dissolved in filtered sea water, and 0.1 ml of a solution was injected into the haemocoel. Since the body volume of *C. limacina* is about 1 ml, the concentration of drugs in the haemolymph was about one-tenth of that which was injected. Effects of injection of each antagonist were tested in at least three experiments.

Two types of preparations were used in electrophysiological experiments ($n$=19), preparations of the isolated central nervous system (CNS), or of the CNS connected with dorsal or ventral wing muscles. Each wing contains two main layers of muscles, dorsal and ventral, with motor nerves passing between the layers. To make a preparation, one of the muscle layers was removed. The preparations were put into a recording chamber lined with agar gel (volume of agar 2 ml). In most experiments preparations were held in place with a thin layer of thickening agar. This method of tethering made it possible to record simultaneously several neurons for many hours. In the CNS-wing muscle preparations, muscle movements were additionally restricted by tethering the muscles with pins. The agar was covered with 2 ml of seawater (see Arshavsky et al. 1985a for details). All drugs were also tested on preparations which were not covered with agar and only tethered by pins.

Intracellular recordings were carried out with 3 M KCl-filled glass microelectrodes having tip resistance of 40–60 MΩ. Sometimes electrodes filled with potassium acetate were used; results obtained were the same using either electrolyte. To facilitate the insertion of microelectrodes, ganglia were treated with Pronase E (Sigma; 1% solution for 3–5 min). Current was injected into neurons through the recording electrode. A bridge circuit was used to compensate partly for the artifact caused by the polarizing current. Wing muscle contractions were recorded with a photocell focused on the edge of the wing. Activities of neurons and wing movements were recorded by means of a pen recorder with a bandwidth of 0–200 Hz. The records have some distortions because of pen vibrations during fast movements.

To test effects of ACh antagonists, the seawater covering the agar layer was replaced by antagonist solutions. Usually drugs were used in concentrations of $10^{-4}$ M. However, in experiments where preparations were located under the agar layer, the exact concentration of the drugs acting on synapses was unknown. To wash out a drug, the microelectrodes were taken out of cells, and the recording chamber was put into a large volume of seawater for several hours. Then the microelectrodes were again inserted into the cells. In most experiments effects of at least three different drugs were tested. Each antagonist was tested in at least ten experiments.

Some experiments were performed on a motor nerve-wing muscle preparation ($n$=14). The motor nerve was stimulated electrically (single pulses of 5 ms in duration) by means of a suction electrode.

## Results

### Peripheral effects of ACh antagonists

In intact *C. limacina,* injection of atropine (0.1 ml, $10^{-3}$ M) produced a total paralysis of wing movements. Injected animals lay down on the bottom of an aquarium without any wing movements. Tail movements were preserved after injection of atropine. Injection of gallamine, *d*-tubocurarine, and α-bungarotoxin did not affect wing movements.

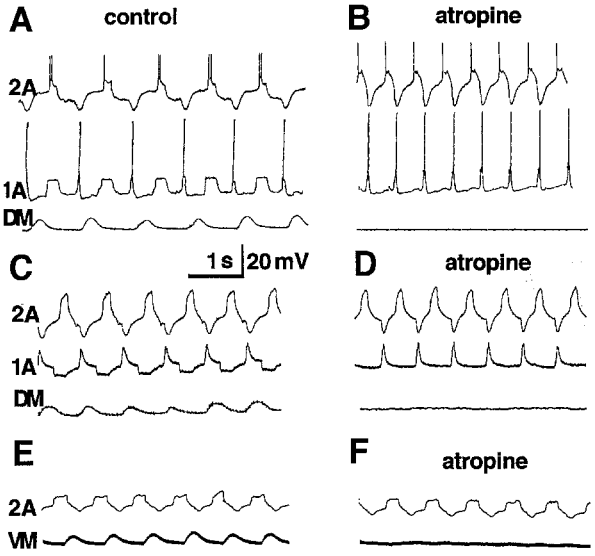


**Fig. 2 A–D** Simultaneous recordings of the 2A and 1A motor neurons and contractions of the dorsal wing muscle (*DM*) before (**A, C**) and after (**B, D**) $10^{-3}$ M atropine application. In **C**, the 1A motor neuron generated the normal IPSPs, while in **A** it generated the inverted IPSPs (see 1* in Fig. 1B). **E, F** Simultaneous recordings of the 2A motor neuron and contractions of the ventral wing muscle (*VM*) before (**E**) and after (**F**) atropine application. Preparations of the CNS connected with the dorsal or ventral wing muscles were used. **A** and **B**, **C** and **D**, and **E** and **F** are from different experiments

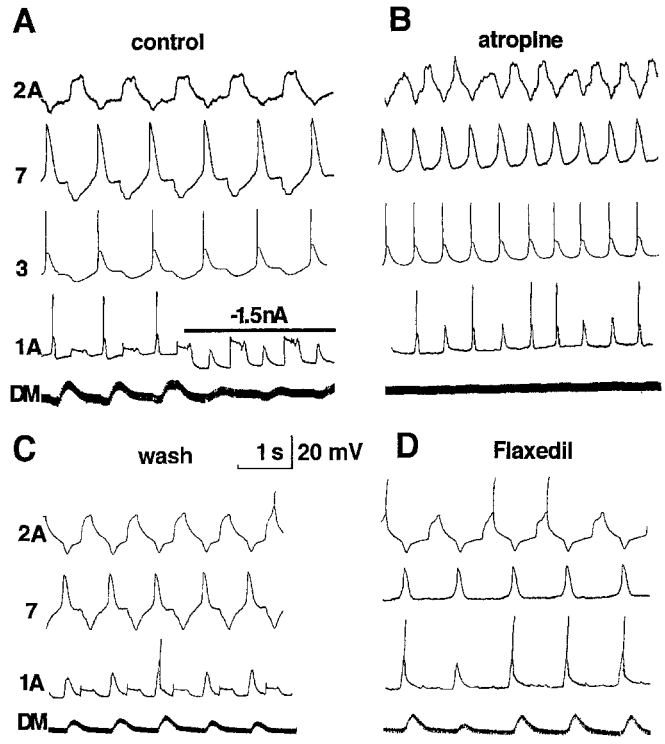


**Fig. 3 A, B** Simultaneous recordings of the 2A and 1A motor neurons, a group 7 interneuron, a group 3 motor neuron, and contractions of the dorsal wing muscle before (**A**) and after (**B**) atropine application. **C** Reversion of the atropine effect upon washout. To wash out atropine, the electrodes were taken out of cells and the recording chamber was put into seawater for 2 h (see Materials and methods). Then the microelectrodes were again inserted into the 2A and 1A motor neurons, and a group 7 interneuron. **D** Effect of gallamine (Flaxedil) application after atropine washout. Preparation of the CNS connected with the dorsal wing muscle. In this and subsequent figures, periods of current injection are marked by *solid lines*, the strength and polarity of the current being indicated near the lines

The same results were obtained in electrophysiological experiments on the CNS-wing muscle preparations (Figs. 2, 3). In Fig. 2A,C the dorsal wing muscle was recorded together with the 1A and 2A motor neurons. In Fig. 2A, the 1A motor neuron generated a single spike in each cycle, muscle contraction following excitation of the motor neuron. After atropine application, muscle contractions disappeared, though periodic EPSPs accompanied by spike discharges persisted in the 1A motor neuron (Fig. 2B). In Fig. 2C, the 1A motor neuron did not produce any spikes, and muscle contractions were therefore determined by spike discharges in other motor neurons active in the D-phase. Muscle contractions disappeared after atropine application (Fig. 2D). The same result was obtained in the preparations of the CNS connected with the ventral wing muscle. In Fig. 2E,F, a mechanogram of the ventral wing muscle was recorded together with the 2A motor neuron. An atropine application blocked muscle contractions (Fig. 2F). In Fig. 3A, the dorsal wing muscle was recorded together with the 1A and 2A motor neurons, a group 3 motor neuron and a group 7 interneuron. Initially, the motor neurons 1A and 3 discharged with single spikes in each cycle, muscle contractions following excitation of the motor neurons. Suppression of spike discharges in the 1A motor neuron by injection of hyperpolarizing current resulted in reduction of muscle contractions. After atropine application (Fig. 3B), muscle contractions totally disappeared, though spike discharges persisted in the motor neurons 1A and 3. Effects of atropine were reversed upon washout (Fig. 3C).

Atropine application also blocked contractions of both dorsal and ventral muscles produced by direct stimulation of the wing nerve in the motor nerve-muscle preparation (Fig. 4). Effects of atropine were reversed (Fig. 4C). Application of gallamine triethiodide, *d*-tubocurarine and α-bungarotoxin (in concentrations up to $10^{-3}$ M) did not influence muscle contractions. An example is shown in Fig. 3D. The dorsal wing muscle was recorded together with the 1A and 2A motor neurons and a group 7 interneuron. Application of gallamine blocking the IPSPs in the neurons 1A and 7 (see below) did not affect muscle contractions.

## Central effects of ACh antagonists

From Figs. 2A–D and 3A,B, it is clear that atropine, in addition to peripheral effects, affected connections between neurons within the neuronal network controlling swimming in *C. limacina*. It blocked the midcycle IPSPs arising in neurons of the D-phase half-center, that is, in the group 7 interneurons (Fig. 3B) and in the group 1

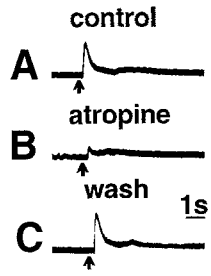


**Fig. 4** **A** Contraction of the dorsal wing muscle produced by a single stimulation of the wing nerve. The wing nerve-wing muscle preparation. **B** Suppression of the muscle contraction after atropine ($10^{-4}$ M) application. **C** Reversion of the muscle contraction after washing in sea water

(Figs. 2B,D and 3B) and 3 (Fig. 3B) motor neurons. IPSPs in group 1 motor neurons were blocked whether they were normal or reversed (cf. Fig. 2A,B and C,D). Postsynaptic potentials in the 2A motor neuron as well as EPSPs in the group 1 and 3 motor neurons did not change.

Figure 5 shows additional examples of central effects of atropine application. In Fig. 5A,B, interneurons of groups 7, 8, and 12 and the 2A motor neuron were recorded simultaneously. Atropine application blocked the midcycle IPSPs in the group 7 interneuron and did not affect potentials recorded in neuron 2A and group 8 and 12 interneurons. From Figs. 3B and 5B, one can see that, after a blockage of IPSPs, discharges of group 7 interneurons were preceded by a ramp depolarization ("pacemaker potential"). Figure 5C and D shows that atropine did not block IPSPs and EPSPs produced by group 7 and 8 interneurons, respectively, onto group 12 interneurons. For exposing IPSPs, the group 12 neuron was depolarized (Fig. 5C), while, for exposing EPSPs it was hyperpolarized (Fig. 5D). Figure 5C also shows that an inhibitory effect of group 12 interneurons on the 2A motor neuron persisted after atropine application.

Although gallamine triethiodide did not affect neuromuscular transmission, it produced the same central effect as atropine, that is, it blocked the midcycle IPSPs arising in the motor neurons and interneurons of the D-phase half-center (Fig. 3D). *d*-Tubocurarine and α-bungarotoxin produced no central effects (not illustrated).

From Figs. 2B,D,F, 3B,D, and 5B,D one could see that the main pattern of the swimming rhythm persisted after elimination of inhibitory connections from group 8 interneurons to antagonistic neurons. Both before and after blockage of these connections, the neurons of D-phase (1A, 3, and 7) and of V-phase (2A, 8, and 12) worked reciprocally with a shift of about half a cycle between two phases. An additional example is presented in Fig. 6A, where the motor neuron 2A and group 7, 8, and 12 interneurons were recorded simultaneously. This figure shows that the main pattern of the activity of the swimming CPG was preserved after elimination of the inhibitory input from group 8 interneurons to the D-phase half-center. One can see that an alternation of two phases took place in spite of the fact that cycle periods changed spontaneously over a rather wide range.

To check how stable the activity of the CPG is after elimination of inhibitory connections from group 8 interneurons to antagonistic neurons, we studied effects of

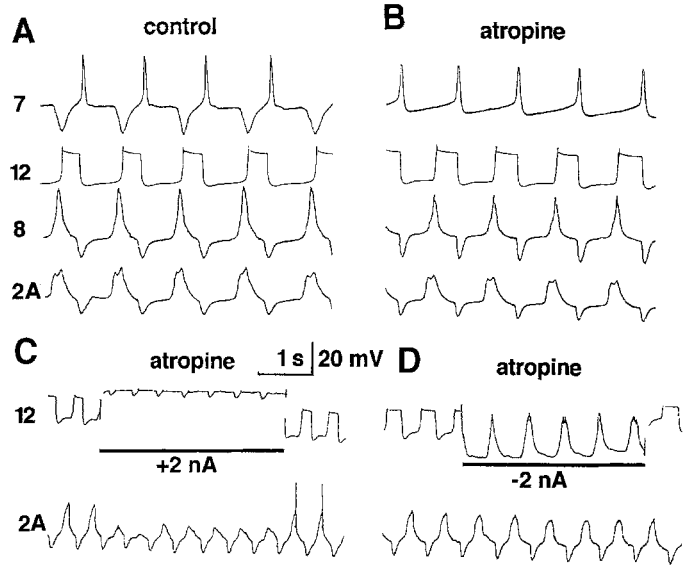


**Fig. 5** **A, B** Simultaneous recordings of group 7, 8, and 12 interneurons, and the 2A motor neuron before (**A**) and after (**B**) atropine application. **C, D** Simultaneous recordings of a group 12 interneuron and the 2A motor neuron after atropine application. Preparations of the isolated CNS were used. **A, B** and **C, D** are from different experiments

neuron polarization on swimming rhythm generation after atropine application. Figure 6B–D shows effects of polarization of the group 7 interneuron on the swimming pattern generated by the CPG after blockage of inhibitory connections from group 8 interneurons to antagonistic neurons. Although a current was injected into a single interneuron, the whole half-center was driven due to strong electrical connections between interneurons of group 7 (see Introduction). Depolarization of the group 7 interneuron resulted in an acceleration of the rhythmic activity but did not change its main pattern as it followed from alternating excitatory and inhibitory potentials in the neuron 2A and the group 8 interneuron (Fig. 6B). The acceleration of the rhythmic activity was determined by shortening of the $V_3$-phase of the cycle, while the D-phase did not change (shortening of the $V_3$-phase is demonstrated most obviously by shortening of a period when the group 12 neuron was in the upper state; see Fig. 1A). A moderate hyperpolarization of the group 7 interneuron resulted in a deceleration of the rhythmic activity accompanied by extension of the $V_3$-phase (Fig. 6C). With stronger hyperpolarization of the group 7 interneuron, the biphasic pattern of the rhythmical activity was broken at least at the beginning of current injection (Fig. 6D). The group 8 interneuron was excited twice in succession before the next excitation of group 7 interneurons occurred (excitation of group 7 interneurons is monitored by IPSPs arising in neurons of the V-phase half-center). Then an alternating activity of two half-centers restarted, probably due to neuron adaptation. The effects of neuron polarization were reproducible and did not depend on spontaneous changes in a frequency of the rhythmical activity.

Figure 7 shows effects of polarization of the 2A motor neuron. Due to electrical connections (see Fig. 1A),

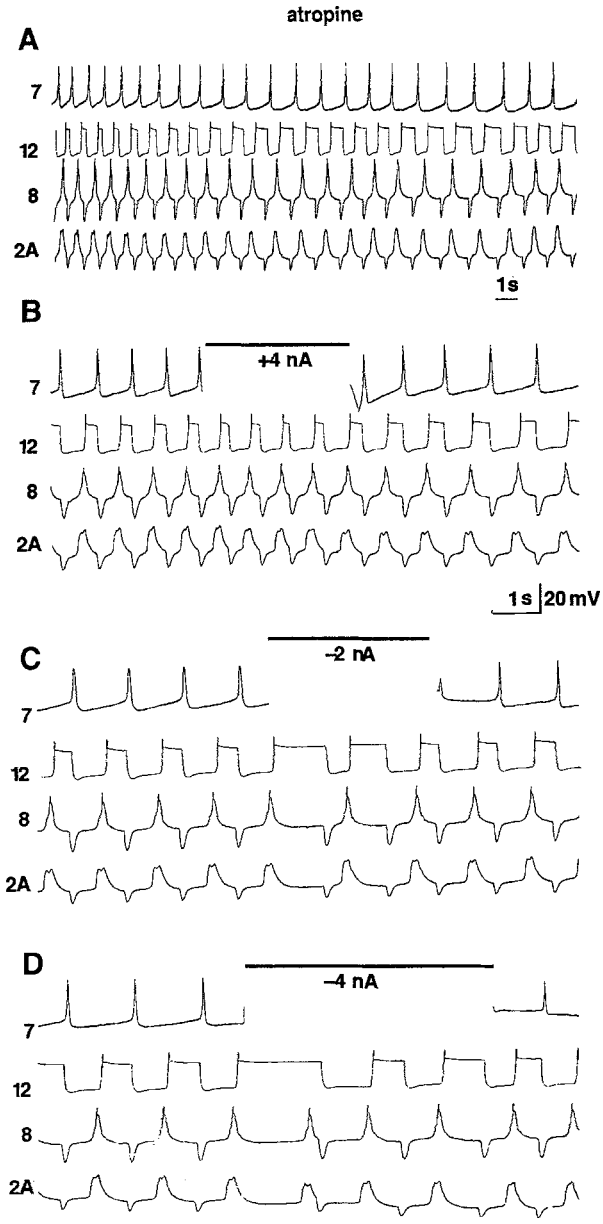


**Fig. 6A–D** Simultaneous recordings of group 7, 8, and 12 interneurons and the 2A motor neuron after atropine application, from the same experiment as in Fig. 5A,B. **A** Initial activity. **B–D** Effects of depolarization (**B**) and hyperpolarization (**C, D**) of the interneuron from group 7 on swimming rhythm generation

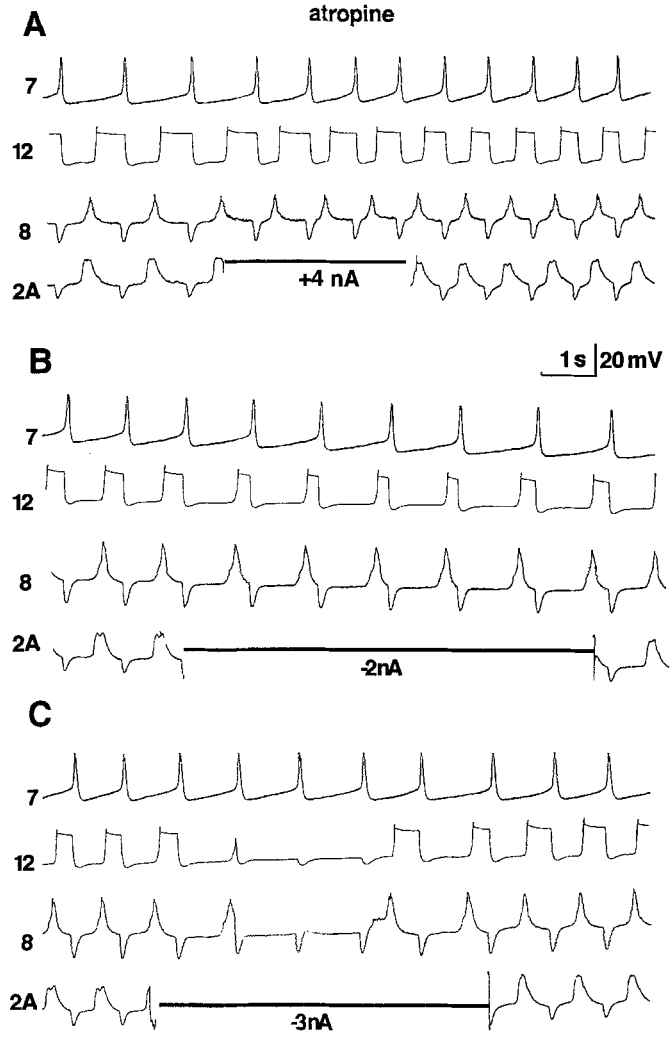


**Fig. 7A–C** Simultaneous recordings of group 7, 8, and 12 interneurons and the 2A motor neuron after atropine application. The same experiment as in Figs. 5A,B and 6. Effects of depolarization (**A**) and hyperpolarization (**B, C**) of the 2A motor neuron on swimming rhythm generation

current injected into the 2A motor neuron, spread out to all neurons of the V-phase, including group 8 interneurons. Injection of depolarizing current (+4 nA) and moderate hyperpolarizing current (up to –2 nA) did not change the general rhythm (within the limits of spontaneous changes of its frequency), and alternation of the D- and V-phases of the cycle persisted. At the same time, current injection affected relations between phases of the cycle (Fig. 7A,B). Injection of depolarizing current enhancing the excitability of group 8 interneurons resulted in shortening of the D-phase of the cycle (Fig. 7A), while injection of hyperpolarizing current resulted in lengthening of the D-phase (Fig. 7B). With increases in hyperpolarizing current injected into the 2A motor neuron, the rhythmical activity of the group 7 interneuron did not change, while the frequency of the activity in the V-phase half-center dropped so strongly that the alternation of two phases was broken (Fig. 7C).

From Figs. 6 and 7 one could see that in cases when the activity of the swimming CPG after atropine application was rather stable, a moderate polarization of the group 7 interneuron affected the general rhythm of the system, while a polarization of the 2A motor neuron did not. This suggests that, after an elimination of inhibitory connections from group 8 interneurons to neurons of the D-phase half-center, group 7 interneurons determine the frequency of a rhythm generation in the system. However, in some cases, when after atropine application rhythm generation was unstable, neurons of the V-phase half-center initiated the swimming cycle. One example is

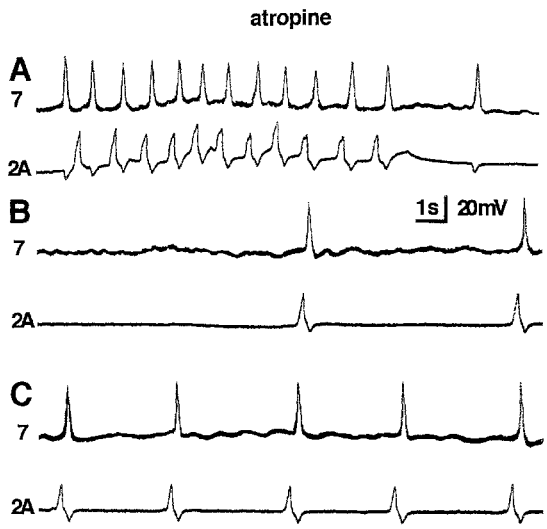


**Fig. 8A–C** Simultaneous recordings of an interneuron of group 7 and the 2A motor neuron after atropine application. Preparation of the isolated CNS. **A** A burst of activity of the swimming central pattern generator (CPG). **B** In the absence of activity of the CPG, spontaneous firings of neurons of the V-phase half-center (as monitored by excitations of the 2A motor neuron) were followed by firings of the group 7 interneuron. **C** Rhythmical activity of the swimming CPG driven by the V-phase half-center

shown in Fig. 8. In this experiment, the activity of the swimming CPG after atropine application was not stable: periods of the rhythmical activity (Fig. 8A) were alternated with periods when the rhythmical activity was practically absent. Such a mode of the swimming CPG operation was observed not only after atropine application but in the untreated isolated pedal ganglia (Arshavsky et al. 1988). When the rhythmical activity of the CPG was practically absent, spontaneous firings of neurons of the V-phase half-center (as monitored by EPSPs in the 2A motor neuron) were immediately followed by firings of neurons of the D-phase which were monitored by excitations of the group 7 interneuron and by IPSPs in the 2A motor neuron (Fig. 8B). Sometimes neurons of the V-phase were excited periodically, driving the rhythmical activity of the system (Fig. 8C). In this case, the transition of excitation from the V-phase half-center to D-phase neurons is believed to be mediated by group 12 interneurons (see Fig. 1A and the Discussion).

## Discussion

### Cholinergic synapses and their pharmacological properties in *C. limacina*

ACh was found to be a transmitter in some peripheral and central synapses of gastropod molluscs (see Walker 1986 for review). In the opisthobranch mollusc *Aplysia californica* the cholinergic efferent neurons exert an excitatory or inhibitory action on the gill muscle (Carew et al. 1974; Weiss et al. 1984), buccal muscles (Church et al. 1993; Cohen et al. 1978; Jordan et al. 1993; Lloyd and Church 1994), the aortic sphincter muscle (Liebeswar et al. 1975), and the heart (Liebeswar et al. 1975). In pulmonate molluscs cholinergic efferent neurons innervate buccal muscles (Cottrell et al. 1983) and the heart (Buckett et al. 1990; Geraerts et al. 1981). Cholinegric interneurons and ACh-sensitive neurons were identified within the CNS of many species of gastropods (Blankenship et al. 1971; Elliott and Kamenes 1992; Elliott et al. 1992; Kandel 1976; Kehoe 1972; King et al. 1987; Morielli et al. 1986; Ter Maat and Lodder 1980; Trudeau and Castellucci 1993; Witte et al. 1985; Yeoman et al. 1993).

Pharmacological analysis has shown that there are many types of cholinergic receptors in gastropod molluscs. Many of these receptors differ radically from "classical" nicotinic and muscarinic receptors described in vertebrates. For example, in one of the first studies of pharmacological properties of cholinergic receptors in *Aplysia californica*, three types of receptors were found (Kehoe 1972). Two types of receptors were blocked by the nicotinic antagonist, curare; one of them was also blocked by the other nicotinic antagonist, hexamethonium. The third type of receptor was not affected by the ACh agonists nicotine and muscarine, and ACh antagonists curare and atropine. This type of receptor was selectively activated by arecoline and blocked by tetraethylammonium. The excitatory effect of ACh upon cholinergic receptors in the *Aplysia californica* gill muscle was reduced by both the muscarinic antagonist atropine and the nicotinic antagonist hexamethonium, though it was not affected by the other nicotinic antagonist *d*-tubocurarine (Weiss et al. 1984). It was also found that the cholinergic receptors in the *Aplysia californica* gill muscle were not sensitive to the ACh agonist carbachol (Weiss et al. 1984). The depolarizing and hyperpolarizing ACh responses of buccal neurons in *Helix pomatia* and *Lymnaea stagnalis* were found to be blocked by both the nicotinic antagonists *d*-tubocurarine and hexamethonium and the muscarinic antagonist atropine (Elliott et al. 1992; Witte et al. 1985). Recently a difference between cholinergic receptors in molluscs and vertebrates was confirmed in a study of effects of α-conotoxins (Fainzilber et al. 1994). It was found that the mollusc-specific α-conotoxins blocking cholinergic receptors in *Aplysia californica* neurons did not influence cholinergic receptors in vertebrates. In contrast, α-conotoxins, which were selective for vertebrates, did not influence cholinergic receptors in molluscs.

In this work it has been found that ACh antagonists block an excitatory action exerted by pedal motor neurons on wing muscles as well as an inhibitory action exerted by group 8 interneurons on neurons of the D-phase half-center. The neuromuscular transmission was blocked by atropine and was not affected by gallamine triethiodide, *d*-tubocurarine, and α-bungarotoxin. IPSPs produced by group 8 interneurons onto neurons of the D-phase half-center were blocked by atropine and gallamine triethiodide, and were not affected by *d*-tubocurarine and α-bungarotoxin. Though atropine blocked both neuromuscular transmission and IPSPs in neurons of the

D-phase half-center, we are not ready to conclude that cholinergic receptors which were under study are muscarinic ones. In a preliminary study we have found that a nicotine application produced both a wing muscle contraction and hyperpolarization of neurons of the D-phase half-center. Thus, additional studies of effects of ACh agonists and antagonists are necessary.

It is interesting that, although atropine and gallamine blocked IPSPs produced by group 8 interneurons onto neurons of the D-phase half-center, they did not affect EPSPs produced by group 8 interneurons onto interneurons of groups 12. There are at least three possible explanations for this result. (1) IPSPs in neurons of the D-phase half-center and EPSPs in group 12 interneurons are produced by different transmitters released by the same group 8 interneurons. It has been shown that, in molluscan cholinergic neurons, ACh is often colocalized with other transmitters such as small cardioactive peptides, FMRFamide, myomoduline, and buccalin (Buckett et al. 1990; Cottrell et al. 1983; Cropper et al. 1990; Schaefer et al. 1985). (2) Cholinergic receptors in inhibitory and excitatory synapses are pharmacologically distinguishable. As a result, ACh antagonists blocking transmission in inhibitory synapses do not influence the transmission in excitatory synapses. (3) Interneurons of group 8 are not a homogeneous group of cells. Only some group 8 interneurons are cholinergic cells exerting an inhibitory action on neurons of the D-phase half-center. Meanwhile, other group 8 interneurons exerting an excitatory action on group 12 interneurons and probably on other neurons of the V-phase half-center are not cholinergic. Additional experiments are necessary to distinguish between these possibilities.

## Swimming rhythm generation after elimination of inhibitory connections from the V-phase to D-phase half-centers

A reductionistic approach has proven to be very fruitful for understanding mechanisms of CPG operations. Surgical transections of anatomical connections between different parts of CNSs, pharmacological blockage of specific synapses, and inactivation of identified neurons were used (for reviews see Arshavsky et al. 1991; Grillner et al. 1993; Harris-Warrick et al. 1992; Jacklet 1989; Roberts and Roberts 1983; Selverston and Moulins 1987).

In this work, a pharmacological blockage of some connections within the network controlling *C. limacina's* swimming was used. It has been found that the main pattern of the swimming motor output with the alternating activity of two half-centers persisted after elimination of the inhibitory connections from the V-phase half-center to the D-phase half-center. Figure 9A shows a scheme of the swimming CPG with inhibitory connections from the V-phase half-center to the D-phase-half center blocked; while Fig. 9B shows the activity of different groups of interneurons in the swimming cycle. As in the case of

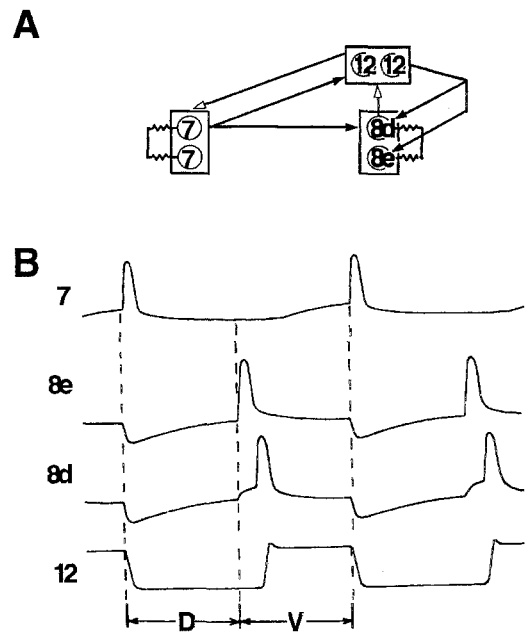


**Fig. 9** **A** The swimming central pattern generator (CPG) with eliminated connections from group 8 interneurons to group 7 interneurons. **B** Phases of the activity of different groups of interneurons after elimination of inhibitory connections from group 8 interneurons to interneurons of group 7. See text for details

the normal activity of the CPG (see Fig. 1B), group 7 interneurons fire periodically, due to their ability to generate the endogenous rhythmical activity and, to some extent, due to the excitatory input from group 12 interneurons (Arshavsky et al. 1985d, 1986, 1989). Group 7 interneurons produce IPSPs onto group 8 interneurons, preventing them from firing in the D-phase of the cycle. With termination of the IPSPs in neurons of V-phase half-center, interneurons of subgroup 8e and then of 8d, are excited. The subgroup 8d interneurons produce EPSPs onto group 12 interneurons, passing them into the upper state. In their turn, group 12 interneurons inhibit neurons of the V-phase half-center, preventing them from repetitive excitation before the beginning of the next cycle. Thus, connections preserved after elimination of the inhibitory input from the V-phase half-center to the D-phase half-center are sufficient for the alternating activity of antagonistic neurons. This result directly contradicts the hypothesis that generation of the swimming rhythmical activity is *exclusively* based on reciprocal inhibition between two half-centers and postinhibitory rebound (Satterlie 1985, 1989).

A shift of about half a cycle between two phases after elimination of the inhibitory input from the V-phase half-center to the D-phase half-center seems to be determined by the fact that intrinsic frequencies of the endogenous rhythms in group 7 and 8 interneurons are similar. This conclusion follows from results obtained in experiments with neuron polarization. From Figs. 6B,C and 7A,B one could see that moderate polarizations of neurons of one of the half-centers, which did not break the alternative activity of antagonistic neurons, changed the structure of

the cycle, that is, relations between two phases. Under depolarization of the group 7 interneuron (Fig. 6B) or hyperpolarization of the 2A motor neuron (Fig. 7B), the V-phase of the cycle became shorter than D-phase. In both cases, under influence of polarization, an intrinsic frequency of the endogenous activity of the D-phase neurons was higher than an intrinsic frequency of the V-phase neurons. As a result, neurons of the D-phase half-center were excited earlier in the cycle than before polarization. On the contrary, under hyperpolarization of the group 7 interneuron (Fig. 6C) or depolarization of the 2A motor neuron (Fig. 7A), the D-phase of the cycle became shorter than V-phase. In these cases under influence of polarization an intrinsic frequency of the endogenous activity of the V-phase neurons was higher than an intrinsic frequency of the D-phase neurons. As a result, neurons of the V-phase half-center were excited earlier in the cycle than before polarization. Under stronger polarization, intrinsic frequencies of the neurons of two half-centers were so different that their alternating activity was broken (Figs. 6D and 7C). It is noteworthy that a stability of the system after elimination of inhibitory effects from the V-phase half-center to the D-phase half-center was lower than in the untreated ganglia. It was previously shown that, in normal saline, moderate de- or hyperpolarization of interneurons of one of the half-centers resulted in an acceleration or deceleration of the swimming rhythm due to shortening/lengthening of both phases of the cycle (Arshavsky et al. 1985c).

The results obtained in this work indicate the persistence of the main pattern of the swimming rhythmical activity after elimination of inhibitory connections from one half-center to the other. This suggests the considerable redundancy of the system controlling *C. limacina's* swimming. Swimming rhythm generation is determined by several factors complementing each other. Similar results were obtained in studying mechanisms of rhythm generation in the crustacean stomatogastric system (Harris-Warrick at al. 1992; Selverston and Moulins 1987) and in the lamprey spinal cord (Grillner et al. 1993). It was found in the stomatogastric system that, after photoinactivation of some neurons or elimination of some inhibitory connections, the pyloric and gastric CPGs continued to produce rhythmical activities, which were altered but still recognizable (for references see Harris-Warrick at al. 1992; Selverston and Moulins 1987). This suggests that in the stomatogasric CPGs there are redundant mechanisms similar to those in *C. limacina.* One can suppose that this redundancy ensures reliable operation of the systems and contributes to their flexibility.

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