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Control of locomotion in marine mollusc *Clione limacina*

VI. Activity of isolated neurons of pedal ganglia

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Summary. In the pteropodial mollusc *Clione limacina*, the rhythmic locomotor wing movements are controlled by the pedal ganglia. The locomotor rhythm is generated by two groups of interneurons (groups 7 and 8) which drive efferent neurons. In the present paper, the activity of isolated neurons, which were extracted from the pedal ganglia by means of an intracellular electrode, is described. The following results have been obtained: 1. Isolated type 7 and 8 interneurons preserved the capability for generation of prolonged (100–200 ms) action potentials. The frequency of these spontaneous discharges was usually within the limit of locomotor frequencies (0.5–5 Hz). By de- or hyperpolarizing a cell, one could usually cover the whole range of locomotor frequencies. This finding demonstrates that the locomotor rhythm is indeed determined by the endogenous rhythmic activity of type 7 and 8 interneurons. 2. Type 1 and 2 efferent neurons, before isolation, could generate single spikes as well as high-frequency bursts of spikes. These two modes of activity were also observed after isolating the cells. Thus, the bursting activity of type 1 and 2 neurons, demonstrated during locomotion, is determined by their own properties. Type 3 and 4 efferent neurons generated only repeated single spikes both before and after isolation. 3. The activity of the isolated axons of type 1 and 2 neurons did not differ meaningfully from the activity of the whole cells. Furthermore, in the isolated pedal commissure, we found units whose activity (rhythmically repeating prolonged action potentials) resembled the activity of type 7 and 8 interneurons. These units seemed to be the axons of type 7 and 8 interneurons. Thus, different parts of the cell membrane (soma and axons) have similar electric properties.

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Key words: Pteropodial mollusc – Pedal ganglia – Locomotion – Interneurons and efferent neurons – Endogenous activity – Isolated cells

Introduction

In the preceding papers of this series (Arshavsky et al. 1985a–e), neuronal mechanisms controlling rhythmic wing movements in the pteropodial mollusc *Clione limacina* were described. The main results of the studies are summarized in Fig. 1. The locomotor rhythm is generated by two groups of interneurons of the pedal ganglia (groups 7 and 8), each of them containing about 10 cells. Type 7 and 8 neurons are small cells with a soma diameter of about 15–20 μm. They have thin neuropilar processes in the ipsilateral ganglion and an axon coming to the opposite ganglion through the pedal commissure. The neurons of each group are electrically connected with each other. The main peculiarity of these neurons is that they generate prolonged action potentials (about 100 ms), one potential per locomotor cycle. Type 7 neurons inhibit type 8 neurons, and vice versa, which determines their alternating activity in the opposite (D and V) phases of the swim cycle. These interneurons drive efferent pedal neurons which send axons to wing muscles. As shown in Fig. 1, interneurons form excitatory and inhibitory chemical synapses as well as electrical synapses on efferent neurons. There are four main groups of efferent neurons: groups 1 and 3 firing in-phase with type 7 interneurons, as well as groups 2 and 4 firing in-phase with type 8 interneurons. Among type 1 and 2 neurons there are two big visually identifiable cells, 1A and 2A. During locomotion, type 1 and 2 neurons generate either single spikes or high-frequency

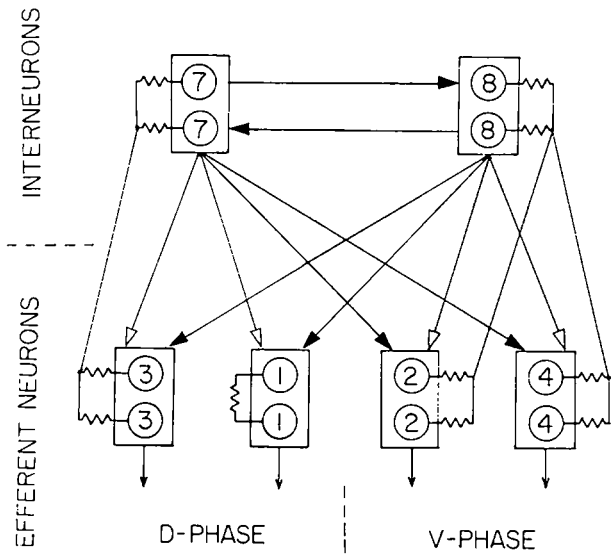

Fig. 1. Locomotor generator of *Clione*. The main groups of neurons exhibiting rhythmic activity during swimming and their connections are shown. (Electric connections are shown by resistor symbols; excitatory and inhibitory connections mediated by chemical synapses are shown by white and black arrows, correspondingly) (Arshavsky et al. 1985c)

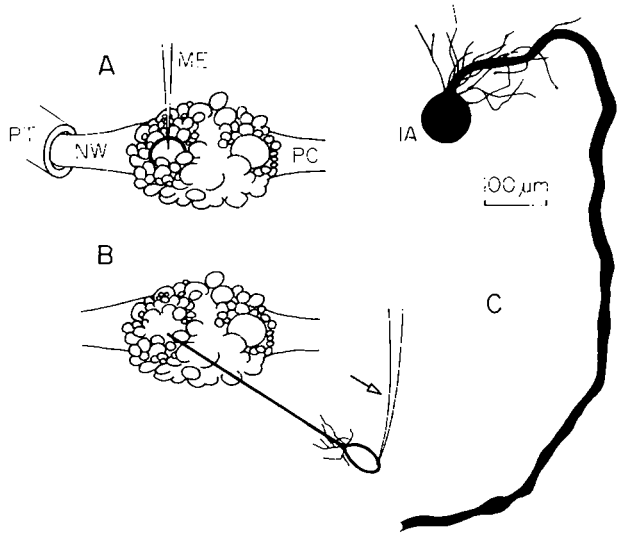

Fig. 2A–C. Method of neuron isolation. **A** Schematic drawing of a pedal ganglion. NW – nerve of the wing sucked into a polyethylene tube (PT), PC – pedal commissure, ME – microelectrode. **B** Extracting of a neuron from the ganglion by means of a microelectrode. **C** The isolated 1A efferent neuron (drowing was made under a microscope after withdrawing the electrode out of the neuron)

bursts. These bursts arise on the top of Π-shaped depolarization. Type 3 and 4 neurons do not generate bursts but single spines only (1–3 per cycle).

When the chemical synapses in the pedal ganglia were blocked by cobalt ions, the rhythmic activity in type 7 and 8 interneurons persisted (Arshavsky et al. 1985c). This led to a hypothesis that generation of the locomotor rhythm in *Clione* is based on the endogenous rhythmic activity of type 7 and 8 interneurons. In the present study, by extracting type 7 and 8 interneurons from the ganglia, we have demonstrated directly that type 7 and 8 interneurons exhibit endogenous rhythmic activity. Besides, we have also studied the activity of isolated efferent neurons and found that the capacity for burst generation in type 1 and 2 neurons persisted after isolation.

Methods

The study was carried out at the White Sea Marine Biological Station "Kartesh" of the Zoological Institute of the Academy of Sciences. Experiments were performed both on isolated neurons (i.e. on cell bodies with their processes) and on isolated axons. Several methods of isolation of identified neurons have been described (London and Merickel 1979; Swidchem 1979; Gillette et al. 1980; Dagan and Levitan 1981; Fuchs et al. 1981; Hadley et al. 1983). In this study we extracted neurons from the pedal ganglion by means of an intracellular microelectrode (Dyakonova 1985). This method provided an opportunity to isolate both visually identifiable neurons and those which could be identified only after recording their activity (Arshavsky et al. 1985b).

Our experimental procedure was as follows. The isolated pedal ganglia of *Clione limacina* were fixed in a chamber, filled with seawater, by two polyethylene tubes into which the wing nerves were sucked (Fig. 2A). The ganglia were treated with a 1% pronase E solution for 20–30 min and then with a 0.3% trypsin solution for 20–30 min at 18–20° C. In the course of the enzymatic treatment, the ganglia were desheathed. For intracellular recording and neuron extraction, glass microelectrodes filled with 2.5–3 M KCl or KAc (tip resistances 20–60 MΩ) were used. One electrode was inserted into a known "rhythmic" neuron for monitoring the phase of the locomotor cycle. Another electrode was inserted into a neuron of the opposite ganglion (Fig. 2A), and the character of the neuron's activity was determined. Then the neuron, together with its processes, was extracted from the ganglion by moving the microelectrode as shown in Fig. 2B. The whole extraction procedure took from several seconds to one minute. Figure 2C shows the isolated 1A efferent neuron. One can see a long axon and many short processes branching out of the soma and the proximal part of the axon. The good condition of isolated neurons is proved by the fact that their membrane and action potentials remained unchanged for many hours.

To isolate an axon, two methods were used. In some cases the second microelectrode was inserted into the axon of an isolated neuron at some distance from the soma. Then, the soma was lifted to the surface of the seawater by the first microelectrode inserted into the soma. Upon emerging from the water, the soma burst. At this moment, the membrane potential recorded from the axon diminished and, after several seconds, was restored, thus indicating the restoration of integrity of the axonal membrane. This method was used for the large 1A and 2A efferent neurons. Another method was used for small type 7 and 8 interneurons. As mentioned above, the interneuron axons go into the pedal commissure where they can be recorded from intracellularly (Arshavsky et al. 1985b). They can be distinguished from the axons of other neurons according to their prolonged action potentials. Therefore, for experimenting with isolated axons of

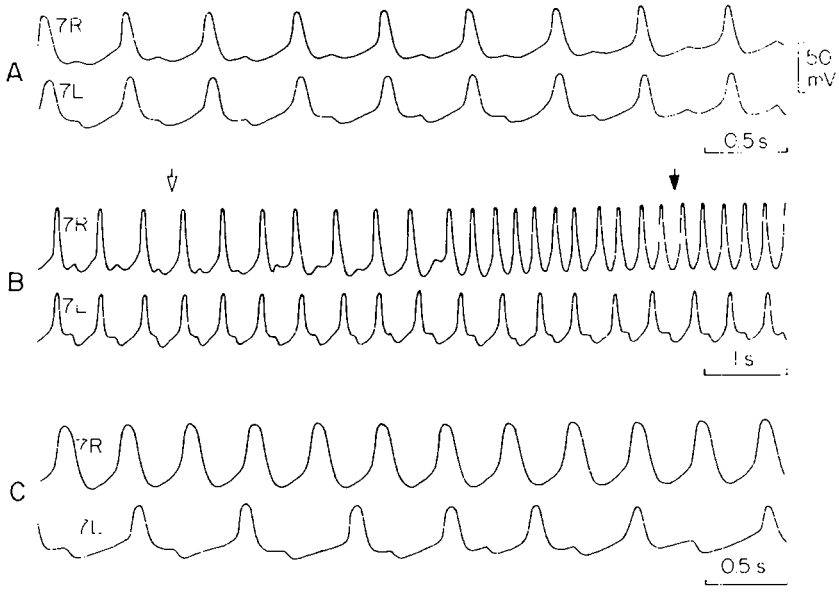

Fig. 3A–C. Isolation of a type 7 interneuron. **A** Initial activity of two type 7 neurons from the right (R) and left (L) pedal ganglia. **B** Extraction of the neuron from the right ganglion. The white arrow marks the beginning of the extraction. The black arrow marks the moment of the total extraction of the neuron. **C** Activity of the type 7 neuron within 10 min after its isolation. The lower trace is the activity of the type 7 neuron which remained in the left ganglion

interneurons, the pedal commissure (which is about 200 μm long) was cut off from both pedal ganglia. To soften the epineural sheath covering the commissure, it was treated with a 1% pronase solution for 5–10 min. Then the commissure was put into a recording chamber lined with agar gel and fixed with a thin layer of agar (for details see Arshavsky et al. 1985a, b). In this method the sole criterion for identification of the recorded units was the duration of their action potentials.

For neuron polarization, current was injected through the recording electrode, a bridge circuit being used. However, bridge compensation was not complete and the records presented in the paper do not allow one to evaluate the actual shifts of the membrane potential in a polarized neuron. For measuring the input resistance, two microelectrodes were inserted into a neuron.

Neuron activity was amplified with a conventional electrophysiological apparatus and displayed on a pen recorder, which resulted in some distortion of the records because of the radial pen movement. The amplitudes of spikes were reduced because of the restricted frequency range (0–200 Hz) of the pen recorder.

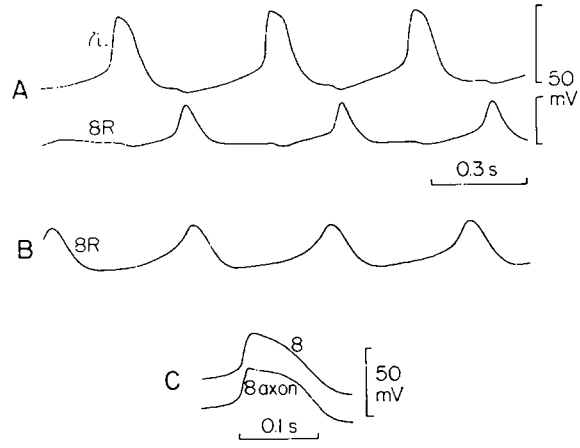

Fig. 4A–C. Isolation of type 8 interneurons. **A** Initial activity of type 7 and type 8 interneurons. **B** Activity of the type 8 interneuron within 15 min after its isolation. **C** Simultaneous recording from the soma and axon of an isolated type 8 neuron (another experiment)

Results

A. Type 7 and 8 interneurons

Ten interneurons were isolated (7 neurons of type 7 and 3 neurons of type 8). Figure 3 illustrates an example of type 7 neuron isolation. Two type 7 neurons from the right and left pedal ganglia were recorded initially (Fig. 3A). One can see prolonged action potentials, which appear in both neurons simultaneously, and mid-cycle IPSPs. In the course of the neuron extraction (Fig. 3B, the upper trace) the IPSPs diminished in amplitude and then entirely disappeared. The discharge frequency of the neuron increased in the course of the extraction due to cessation of the inhibitory input and, probably, to some deformation of the neuron membrane. Within 20–40 s after extraction, the neuron discharge frequency decreased somewhat and then remained at a constant level for many hours (Fig. 3C). That kind of activity was observed in five isolated type 7 interneurons. In two neurons, the activity disappeared after isolation, but it could be restarted by injecting weak depolarizing current (see below).

Isolated type 8 neurons also generated rhythmic action potentials for many hours (Fig. 4A, B). In one case, we succeeded in inserting 2 microelectrodes into the soma and axon of an isolated type 8 neuron (Fig. 4C). Action potentials in the soma and axon arose practically simultaneously and had a similar

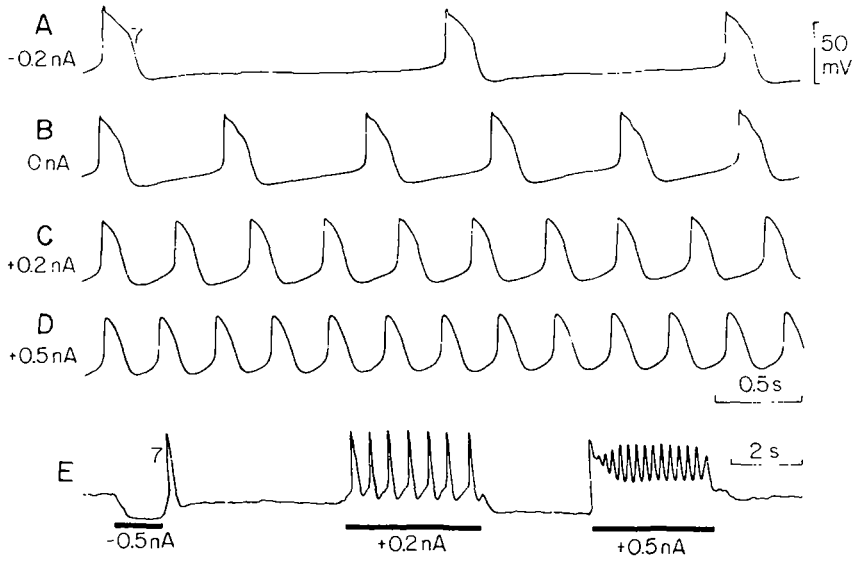

Fig. 5A–E. Effects of polarization on the activity of isolated type 7 interneurons. **A–D** Polarization of a spontaneously active neuron. In **A, C, D** polarization was started before the beginning of the record, the polarity and current strength being indicated on the left. **E** Polarization of another neuron without a spontaneous activity. Periods of current injection are marked by solid lines.

shape and duration. One can see from Figs. 3C and 4B that during the process of rhythmic generation each discharge of a neuron was preceded by a smoothly increasing depolarization (see also Figs. 5A–D, 6A–C).

The discharge frequency of isolated interneurons depended on the level of the membrane potential. Neuron hyperpolarization resulted in a decrease of the discharge frequency (Fig. 5A) while depolarization, in its increase (Fig. 5C, D). By de- and hyperpolarizing a neuron, we could usually cover the whole range of the locomotor frequencies (0.5–5 Hz). Type 7 and 8 neurons exhibited no adaptation to injected current: the discharge frequency corresponding to a given current could persist for several hours as long as the current was passed.

In the cases when rhythmic discharges stopped after extracting a neuron from the ganglion, the rhythmic activity could be restarted by depolarizing the neuron (Fig. 5E). The current strength needed for this was not above 0.5 nA. In three cases we managed to insert two microelectrodes into an interneuron and to measure its input resistance which was 15–20 MΩ. This means that the shifts of membrane potential needed for restarting spontaneous discharges of "silent" interneurons were within 10 mV.

In "silent" interneurons, a rebound phenomenon was observed. As shown in Fig. 5E (left), after termination of the hyperpolarizing current an action potential was generated. The strength of current sufficient to obtain postinhibitory rebound was within 0.5 nA.

After extraction from the ganglion, action potentials of interneurons were usually somewhat extended (Figs. 3 and 4). In some experiments, the

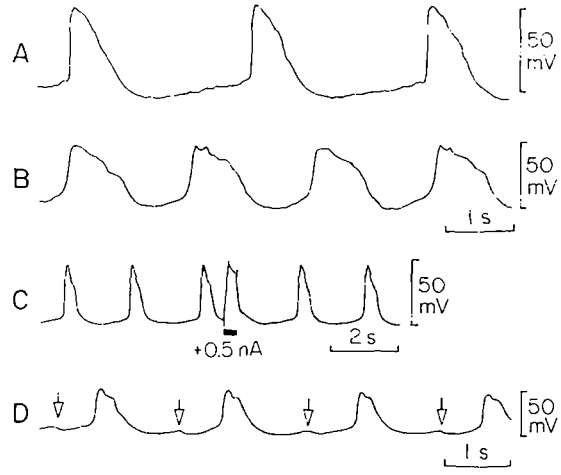

Fig. 6A–D. Recording of units in the isolated pedal commissure (putative axons of type 7 and 8 interneurons). **A** Initial activity of an axon. **B** Depolarization of the axon (current of 0.5 nA). **C** Extra-excitation of the axon by a short pulse of depolarizing current. **D** Another experiment. Arrows mark the mid-cycle IPSPs

duration of the action potentials changed inversely to the discharge frequency (Fig. 4A–D). Note that in non-isolated interneurons the duration of action potentials did not depend on the locomotor frequency (Arshavsky et al. 1985c).

In experiments on the isolated pedal commissure, we succeeded in recording three units which seemed to be axons of type 7 or 8 interneurons since they generated prolonged action potentials (Fig. 6). These units also discharged periodically with a frequency similar to the locomotor one (Fig. 6A). The discharge frequency increased with the depolarization of axons (Fig. 6B). Extra-excitation of an axon

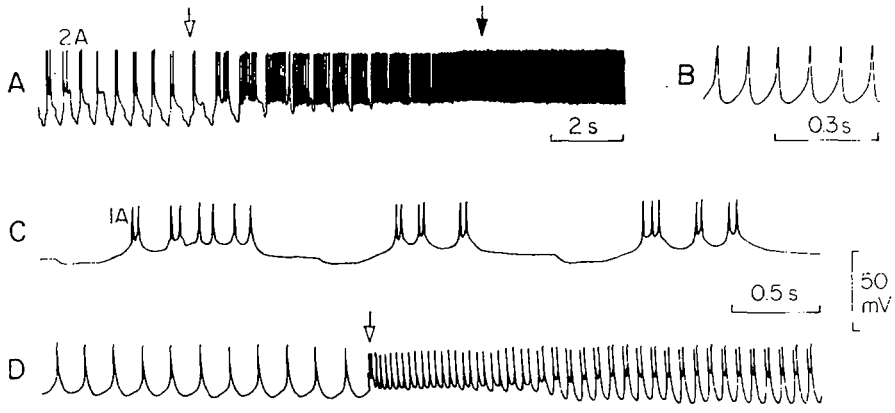

Fig. 7A–D. Isolation of the 1A and 2A efferent neurons. **A** Extraction of the 2A neuron from the pedal ganglion. The beginning of the extraction is marked by the white arrow, and the finish, by the black one. **B** Activity of the 2A neuron just after its isolation. **C, D** Isolation of the 1A neuron. Initial activity (**C**) and activity within 30 s after isolation (**D**) are shown. The arrow in **D** marks the beginning of neuron depolarization (current of 3 nA)

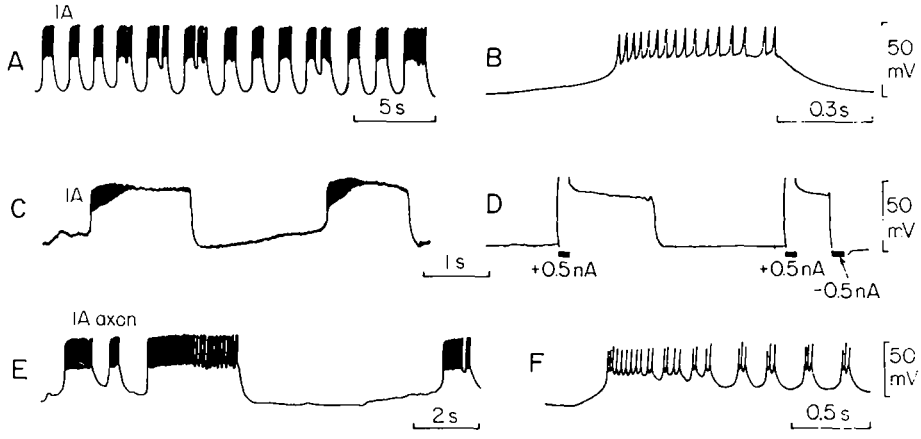

Fig. 8A and B. Bursting activity of the isolated 1A neuron taken on two different time bases. **C, D** "Plateau" potentials generated by the isolated 1A neurons. Black rectangles in **D** mark periods of current injection, the polarity and current strength being indicated near the rectangles. The recordings **C** and **D** are from different experiments. **E, F** Bursting discharges of an isolated axon of the 1A neuron taken on two different time bases

by a short pulse of depolarizing current resulted in a shift of the phase (Fig. 6C).

In one of the putative interneuron axons we observed small IPSPs between action potentials (marked by arrows in Fig. 6D). This suggests that an inhibitory interaction between antagonistic interneurons takes place not only in the neuropile of the ganglia but in the pedal commissure too.

B. Efferent neurons

Twenty-eight efferent neurons were isolated (16 neurons of type 1, including 12 neurons of 1A, 7 neurons of 2A, two neurons of type 3 and three neurons of type 4). Figure 7 illustrates examples of isolation of 2A (A, B) and 1A (C, D) neurons. One can see that in the course of extraction, IPSPs in the 2A neuron decreased and then disappeared (Fig. 7A). At the same time, the discharge frequency of the neuron increased which could be explained by cessation of the inhibitory input and by some deformation of the membrane. The same events were observed during extraction of the 1A neuron (Fig. 7C, D).

The 2A and 1A neurons, shown in Fig. 7, discharged after isolation with single spikes. However, neurons of these types could also discharge in bursts. Figure 7D shows that during depolarization of the 1A neuron, its discharge frequency increased, and then the mode of the neuron activity changed. In this mode, the neuron periodically generated slow waves of depolarization, and on the top of each wave a pair of spikes arose. The bursting activity of the isolated 1A and 2A neurons could also arise spontaneously without injecting depolarizing current (Fig. 8A, B).

Sometimes the isolated 1A neurons generated "plateau" potentials (Fig. 8C, D). The neurons could pass to the depolarized state either spontaneously (Fig. 8C) or after application of a depolarizing current (Fig. 8D). At the beginning of the "plateau", high frequency spike discharges could arise (Fig. 8C). The neurons remained in a depolarized state for 1–3 s, and then the potential returned to the

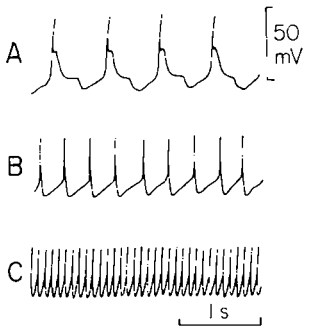

Fig. 9A–C. Isolation of a type 3 efferent neuron. **A** Initial activity of the neuron. **B** Activity within 60 s after isolation. **C** Depolarization of the isolated neuron (current of 1 nA)

initial level. The return of the potential could also be caused by the hyperpolarizing current (Fig. 8D, right).

Bursting discharges arising on the top of slow waves of depolarization were also recorded in isolated axons of the 1A and 2A efferent neurons (Fig. 8E, F).

Unlike type 1 and 2 neurons, isolated type 3 and 4 efferent neurons generated only repeating single spikes (Fig. 9). Depolarization of these neurons resulted in an increase of the spike frequency (Fig. 9C) but caused neither slow oscillations of the membrane potential nor bursting discharges.

Discussion

The main result of this study is that isolated type 7 and 8 interneurons appeared to be capable of endogenous rhythmic activity. They generated prolonged action potentials (about 100 ms) similar to those they generated before extraction from the ganglia. By varying the membrane potential in isolated interneurons within physiological limits (by passing the current through a microelectrode), one could cover the whole range of locomotor frequencies (0.5–5 Hz). This finding supports the hypothesis that the locomotor rhythm in the pedal ganglia arises mainly due to the endogenous rhythmic activity of type 7 and 8 interneurons (Arshavsky et al. 1985c). Synchronous discharge of neurons within the group during locomotion (see e.g. Fig. 3A) can be easily explained by electrical connections between them (Arshavsky et al. 1985b).

A strong dependence of the discharge frequency on the membrane potential, demonstrated in the isolated type 7 and 8 interneurons, explains why the polarization of these neurons in the ganglia influenced the locomotor rhythm. However, to change the rhythm in the whole ganglion, one had to pass through a neuron considerably stronger current (a few nA) than in the case of isolated cells. This agrees well with the fact that in the ganglia each interneuron is a part of a system consisting of about 10 electrically interconnected cells. Besides, interneurons have electrical connections with efferent neurons (Fig. 1). As a result, the input resistance of an interneuron in the ganglion must be smaller than that of an isolated interneuron.

It was demonstrated (Arshavsky et al. 1985c) that rhythmic generation in the pedal ganglia could be evoked even in the case when the membrane potential of type 7 and 8 interneurons was under the threshold of endogenous rhythmic activity. For example, in a quiescent preparation a series of locomotor cycles could be evoked by passing a short pulse of hyperpolarizing current through a type 7 (or 8) interneuron. It seems likely that in such a case, the rhythmic activity was maintained due to the phenomenon of postinhibitory rebound (Arshavsky et al. 1985c; Satterlie, 1985). One can imagine that termination of the hyperpolarizing current passed through an interneuron results in excitation of neurons of a given group which, in their turn, evoke IPSPs in interneurons of the antagonistic group (Fig. 1). After termination of IPSPs, excitation of these antagonistic neurons occurs as a result of their postinhibitory rebound, and so on. The present study has demonstrated that type 7 and 8 interneurons indeed have a strongly pronounced property of postinhibitory rebound (Fig. 5E).

Thus, the properties found in the isolated type 7 and 8 interneurons (the endogenous rhythmic discharges with a period depending on the membrane potential, as well as the postinhibitory rebound) together with the system of mutual inhibitory connections (Fig. 1) are sufficient for explaining the operation of the rhythm generator in the pedal ganglia of *Clione*. To transfer this system from the state of quietness into the state of rhythmic oscillations, it is not necessary to change any property of the membrane of type 7 and 8 interneurons – one has only to depolarize the membrane to an appropriate level. In this respect the *Clione* locomotor generator differs, for example, from the spinal locomotor generator of the lamprey. In the latter, the cells responsible for rhythmic generation have no generator properties at resting conditions, i.e., the rhythmic generation cannot be evoked by merely depolarizing the membrane. Generator properties appear in these cells only after application of a specific mediator responsible for the elicitation of locomotion (Grillner et al. 1983; Sigvardt et al. 1985).

It should be noted that endogenous neuron activity seems to be the basis of rhythmic generation

in some other invertebrates: in the pyloric generator of the lobster stomatogastric ganglion (Maynard and Selverston 1975; Selverston and Miller 1980; Miller and Selverston 1982a, b), in the feeding system of the snails *Helisoma trivolvis* (Merickel and Gray 1980) and *Lymnaea stagnalis* (Rose and Benjamin 1981), in the heartbeat system of the leech (Calabrese 1979; Stent et al. 1979) and of the crab (Tazaki and Cooke 1979a, b), etc. (see Friesen and Stent 1978).

During locomotion, different types of efferent neurons in the pedal ganglia of *Clione* had different kinds of electrical activity: type 3 and 4 neurons discharged only by single spikes while type 1 and 2 neurons could also generate high-frequency bursts. These peculiarities of the discharges persisted in the cells after their extraction from the ganglia and are, therefore, determined by the intrinsic membrane properties. Thus, the efferent pattern of locomotion can be obtained by directing the rhythmic commands to the efferent neurons from type 7 and 8 interneurons, as shown in Fig. 1, without changing any properties of the membrane of efferent neurons.

The membrane of efferent neurons (and, seemingly, that of interneurons) is homogeneous with respect to the electrical properties. This follows from the fact that the activities of isolated axons of efferent neurons as well as of putative axons of interneurons did not differ from the activities of the whole cells.

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