The Journal of Experimental Biology **198**, 905–916 (1995)
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SEROTONERGIC MODULATION OF SWIMMING SPEED IN THE PTEROPOD MOLLUSC *CLIONE LIMACINA*

II. PERIPHERAL MODULATORY NEURONS

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Accepted 12 December 1994

Summary

A symmetrical cluster of serotonin-immunoreactive neurons in the pedal ganglia of *Clione limacina* has been described morphologically and physiologically. At least five of the cluster neurons send axons to the ipsilateral wing that branch throughout the entire wing area. Activation of these cells did not produce a motor effect in non-swimming preparations, but did enhance contractility in swimming preparations. Activity in the pedal neurons did not produce detectable central effects as neither swim interneuron nor swim motor neuron activities were altered. Most notable was a lack of a change in swim frequency, a characteristic of swim acceleration. Activity in the pedal neurons did enhance the size of muscle junctional potentials and spike-like responses, but only in slow-twitch muscles. The peripheral modulatory effect was blocked by the serotonin antagonist mianserin.

Key words: serotonin, swimming, modulation, muscle, mollusc, *Clione limacina*.

Introduction

Increases in locomotory speed can be achieved in several ways, most notably *via* an increase in the frequency of locomotory appendage movements or through an increase in the force of appendage contractions. Additionally, biomechanical changes, such as changes in the angle of attack of wing-like structures, can produce changes in locomotory speed. In the pteropod mollusc *Clione limacina*, the change from slow to fast swimming involves both an increase in wing-beat frequency and an increase in the force of wing contractions (Arshavsky *et al.* 1985*b*,*c*; Satterlie, 1991*a*). Changes from slow to fast swimming that are identical to those observed in spontaneously active preparations can be induced by bathing whole animals or reduced preparations in physiological concentrations of serotonin (10^{-4} to 10^{-6} mol l^{-1}; Arshavsky *et al.* 1985*a*; Satterlie, 1989; Kabotyansky and Sakharov, 1990). Since serotonin immunoreactivity has been demonstrated in several central neurons in the *Clione limacina* nervous system, as well as in the wings, it is worthwhile determining which, if any, immunoreactive neurons are capable of producing either an increase in swim frequency or an increase in wing contractility or both. In this quest, the three primary targets for modulation are the interneurons of the swim pattern generator, swim motor neurons and the swim musculature.

Changes in swim frequency probably involve changes in pattern generator activity, while changes in wing contractility can result from changes in the activity of the swim musculature directly or through modification of motor neuron activity. The primary aims of this and the following paper (Satterlie and Norekian, 1995) are to describe electrophysiologically the serotonin-immunoreactive neurons that produce alterations of activity at these three levels of the locomotory system and to initiate detailed analyses of the role of these cells in both the change from slow to fast swimming and in more subtle changes of swimming speed.

The locomotory system of *Clione limacina* is well suited for such investigations since a great deal is known about the organization of each of the three levels of the swimming system: the pattern generator, the motor neurons and the musculature (Arshavsky *et al.* 1985*a*–*d*,; Satterlie *et al.* 1985, 1990; Satterlie and Spencer, 1985; Satterlie, 1991*a*,*b*, 1993). The pattern generator consists of two groups of antagonistic interneurons (approximately 10 neurons in each group) that interact *via* reciprocal inhibitory connections. The change from slow to fast swimming involves an increase in cycle frequency that is accompanied by, and possibly triggered by, the addition of the activity of two previously inactive interneuron types to the pattern generator (pattern generator reconfiguration; Arshavsky

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et al. 1989). At the neuromuscular level, the change involves the recruitment of a pair of large motor neurons (general excitors, cells 1A and 2A of Arshavsky *et al.* 1985*a–c*) which, in turn, activate fast-twitch fatigable muscle cells of the swim musculature, thus increasing the force of wing contractions. In the present paper, a cluster of serotonin-immunoreactive neurons in the pedal ganglia is investigated electrophysiologically. These cells were initially selected because of their large size (up to 50 μm in diameter) and because the axons of some of these cells clearly extended to the swim musculature. The pedal cells produce enhancement of muscle contractility without increasing swim frequency, suggesting that their primary effects are strictly peripheral. It has been suggested that the serotonergic modulatory system of *Clione limacina* is compartmentalized rather than widespread and diffuse.

Materials and methods

Animal collection, maintenance and dissection are as described previously (Satterlie *et al.* 1995). Intracellular recordings were made with microelectrodes filled with $2\,\text{mol}\,\text{l}^{-1}$ potassium acetate with resistances of 10–30 MΩ. Standard amplification and display electronics were used. Current injection was achieved *via* the recording electrode with an amplifier bridge circuit. To test for monosynaptic connections, preparations were bathed in sea water containing 2.5 times the normal calcium and magnesium concentrations (high-Ca^{2+}, high-Mg^{2+} saline), which increases the threshold of all cells and thus decreases the possibility of conduction in pathways involving more than one chemical synapse.

For uncalibrated force transducer recordings, a glass hematocrit capillary tube was pulled over an alcohol flame to a fine point and fire-polished to slightly dull and seal the tip. The capillary was then fastened to a 400A force transducer system (Cambridge Technology Inc.) with molten wax. The capillary with attached transducer head was then positioned with a micromanipulator so that the capillary tip touched the wing tissue with enough tension to allow the capillary tip to move with the wing tissue without slipping. With this type of placement, the transducer–capillary system acted as a movement detector that was sensitive enough to record the slight contractions of small regions of the swim musculature following stimulation of individual small motor neurons. Since this transducer system was uncalibrated, it could only be used to show relative changes within a single trial. In simultaneous intracellular recordings of wing movements from muscle cells in the immediate vicinity of the transducer capillary, the size of the muscle electrical responses mirrored the size of the force recordings (Fig. 1). This suggests that relative changes in recorded muscle contraction (from several local muscle bundles) reflected the underlying changes in muscle electrical activity from single cells in the same region. Since the capillary tip was not firmly attached to the wing tissue, and the exact angle of the transducer to the tissue was not constant from preparation to preparation, the polarity of the muscle responses was variable so that contractions appeared as downward deflections in some recordings, upward deflections in others and even as biphasic waves in a few recordings.

Results

In the previous paper (Satterlie *et al.* 1995), immunohistochemical and dye-injection data indicated that up to five 5-HT neurons from each pedal ganglion (hereafter called Pd-SW neurons) send axons to their ipsilateral wing and innervate the swim musculature. Despite this morphological evidence for innervation, Pd-SW neurons did not produce a motor effect in the absence of existing swimming activity. Even intense bursts of activation (within the physiological range of up to 40 Hz) during periods of swimming quiescence did not produce any contractile response of the wings. When similarly stimulated during existing swimming activity, however, an enhancement of contractile responses was observed (Fig. 2). This apparent modulatory response appeared as an increase in the size of individual contractions with a latency of 1 s after initiation of a burst of activity in a Pd-SW cell. The duration of the modulatory response was between 2 and 10 s following Pd-SW cell bursts lasting 1.5 s. In all cases, regardless of burst duration, the modulatory effects outlasted the period of Pd-SW cell firing. The morphology of Pd-SW neurons not only suggests the possibility of peripheral modulation of the swim musculature (Satterlie *et al.* 1995), but also that there may be central modulation of swim interneurons and/or motor neurons since the Pd-SW cells contribute numerous processes to the pedal neuropil. Following the initial description of the firing characteristics of these cells, an effort was therefore made to separate the central and peripheral effects of Pd-SW cell activity.

Firing characteristics of Pd-SW cells

Electrophysiological identification of Pd-SW neurons was

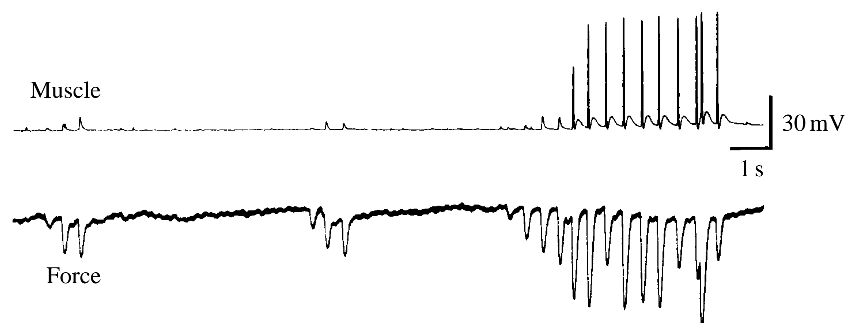

Fig. 1. Relationship between wing contractions and intracellularly recorded electrical activity of a swim muscle cell. The force transducer was placed as close to the recorded cell as possible without disturbing electrode penetration. The strength of wing contraction varied with the amplitude of muscle cell junctional potentials and spike-like responses, suggesting that muscle electrical activity can be used as an estimate of at least regional contraction of the wing.

aided by the observation that all the cells in the cluster showed the same pattern of electrical activity. When the preparation was actively swimming, Pd-SW cells showed irregular action potential activity (Fig. 3). Action potentials were large (up to 70 mV) and up to 8 ms in duration with distinct after-hyperpolarizations. The overall frequency of firing increased when swim frequency increased, and firing rate was significantly decreased and usually stopped altogether when swimming ceased (Fig. 3). When swimming was intermittent, a tonic depolarization, originating as a barrage of EPSPs, was frequently evident at the time of swim initiation (see Fig. 3A). The tonic depolarization continued for the duration of swimming activity and disappeared when swimming ceased. In such intermittently swimming preparations, cessation of swimming was usually accompanied by active inhibition of swim interneurons and swim motor neurons, as shown by barrages of IPSPs in these cells (Fig. 3C). Double recordings from Pd-SW neurons and swim interneurons or motor neurons confirmed that, during such periods of active inhibition, the Pd-SW neurons were similarly inhibited, although individual IPSPs were not clearly visible in most recordings (Fig. 3C). From these recordings, it was not possible to determine

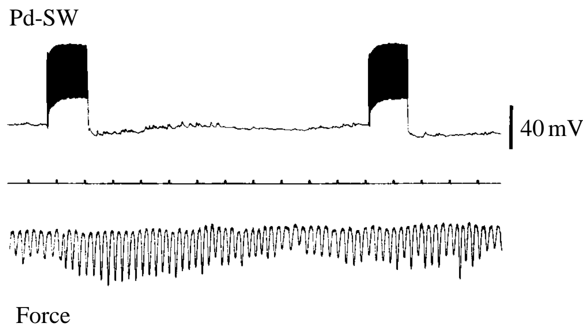

Fig. 2. Modulatory effect of Pd-SW neurons. Induced spike bursts in a Pd-SW neuron, near the maximum natural firing frequency of the cell, trigger increases in the strength of wing contractions that appear with a latency of approximately 1 s and which outlast the spike burst by up to 10 s. Middle trace, 1 s markers.

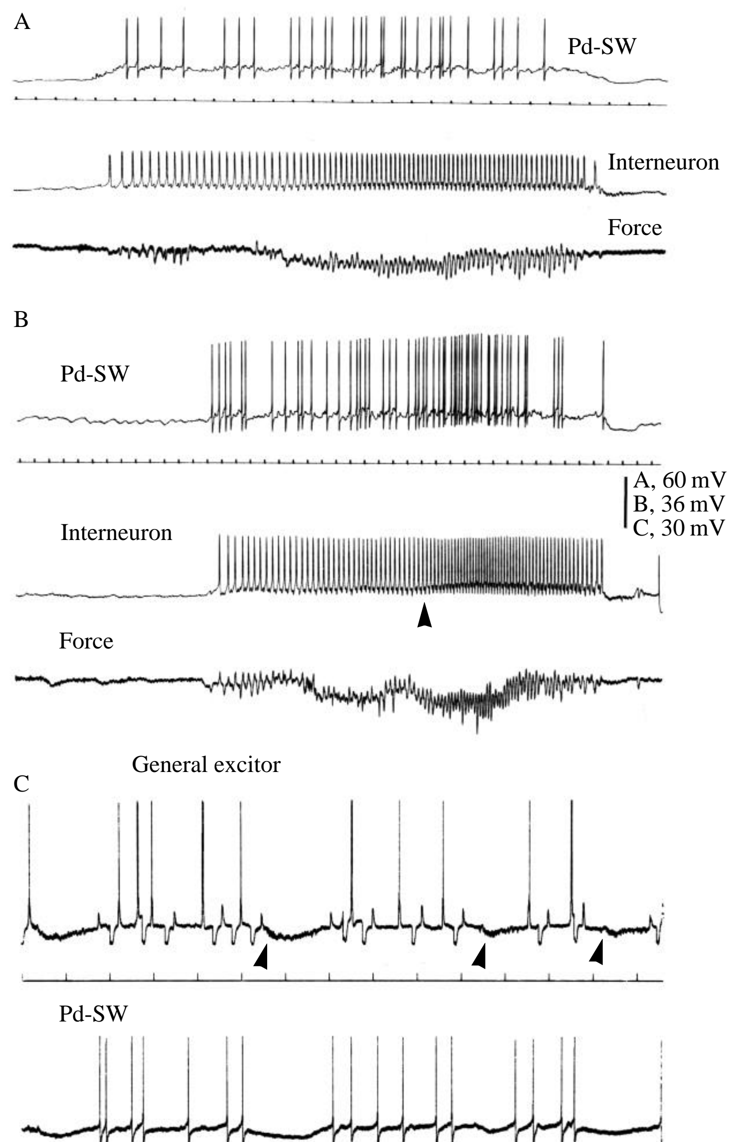

Fig. 3. Spontaneous activities of Pd-SW neurons. (A) Initiation of swimming, as noted by the activity of a swim interneuron, is accompanied by a tonic depolarization of the Pd-SW neuron. The depolarization lasts for the duration of the swimming bout. Note the slight increase in firing frequency in the Pd-SW neuron when swimming speed increases. (B) A sudden increase in swimming speed, seen in the swim interneuron recording at the arrowhead, is accompanied by a significant increase in firing frequency in the Pd-SW neuron. (C) Inhibition of swimming is seen as a barrage of IPSPs in a general excitor motor neuron (arrowheads). Pd-SW neurons are similarly inhibited; however, discrete synaptic potentials are not seen. The tick interval on the time traces is 1 s.

whether the inhibition of Pd-SW neurons was direct or whether their tonic depolarizing inputs were blocked.

Recordings from pairs of Pd-SW neurons indicated that they all produced similar, but not identical, firing activity (Fig. 4). Incoming synaptic potentials were usually common to all neurons, although the production of action potentials varied in different cells (Fig. 4A). Nonetheless, swim acceleration was always accompanied by an increase in firing frequency in all Pd-SW neurons, including those whose axons ran to targets other than the wings. Pd-SW neurons were not electrically coupled to one another. Furthermore, induced bursts of action potentials in one cell did not produce any response in other neurons of the group (Fig. 5).

Mechanical stimulation of a wing produced two types of behavioral responses in intact animals. Mild touches to a wing typically produced a more forceful subsequent contraction or set of contractions of the ipsilateral wing. This response was sometimes accompanied by a similar reaction in the contralateral wing, and on a few occasions, a slight acceleration of swimming speed was noted. Continued mild stimulation of a wing, or strong individual stimuli, typically resulted in withdrawal of the ipsilateral wing, or of both wings, into the body with simultaneous inhibition of swimming. Furthermore, strong stimulation of the tail of intact animals triggered escape responses that included increases in both the strength and frequency of swim contractions. In dissected preparations, mild stimulation of a wing, similar to that resulting in increased force of contraction in intact preparations, produced a rapid burst of action potentials in Pd-SW neurons. Maximal burst variables were 1 s duration at a top frequency of 40 Hz. Similar bursts of action potentials could be triggered by stimulating the head or tail of minimally dissected animals (Fig. 6A,B).

Central effects of Pd-SW cell activity

Changes in the strength of wing contractions can be produced through several central mechanisms, coincident with an increase in the frequency of swim contractions (and a resultant facilitation of muscle responses). These mechanisms include direct synaptic inputs to interneurons and/or motor neurons of the swimming system and modulation of interneuron and/or motor neuron activities, such as spike duration of interneurons or spike frequency of motor neuron bursts. Such interactions were tested between Pd-SW neurons and three classes of swim neurons: pattern generator interneurons, general excitor motor neurons and small motor neurons (see Satterlie, 1993, for a description of motor neuron types).

Initial recordings from Pd-SW neurons indicated that their firing frequency increased when the swim frequency increased. To determine whether the increased swim frequency was triggered by Pd-SW cell activity, the frequency of spontaneous swimming activity was measured before and 2 s after induced

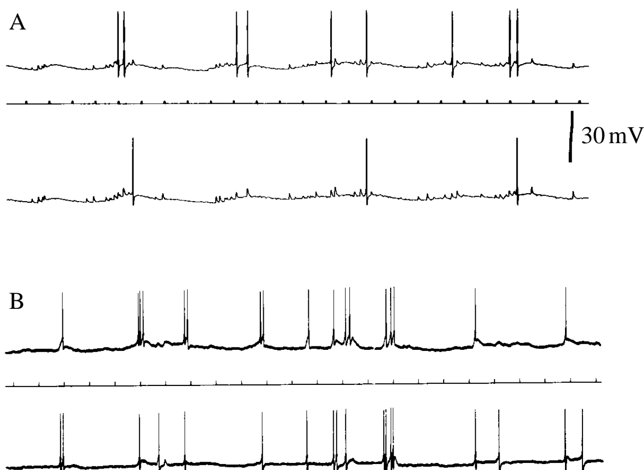

Fig. 4. Pairs of Pd-SW neurons show similar firing activities. (A) Many EPSPs are common to pairs of Pd-SW neurons. (B) Action potential activity is not synchronous, but is very similar in pairs of Pd-SW neurons. The tick interval on the time traces is 1 s.

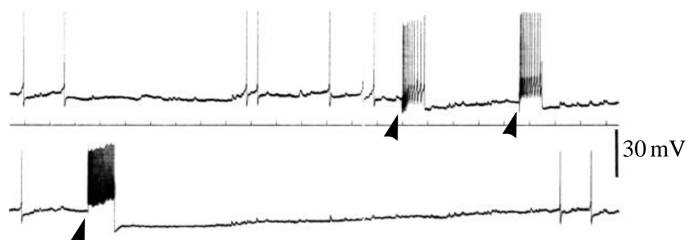

Fig. 5. Pd-SW neurons are not synaptically or electrically coupled. Initiation of spike bursts with depolarizing current injection in one cell (arrowheads) does not alter the membrane potential of other Pd-SW neurons. The tick interval on the time trace is 1 s. Injected currents, 0.6 nA in the top trace and 1.1 nA in the bottom trace.

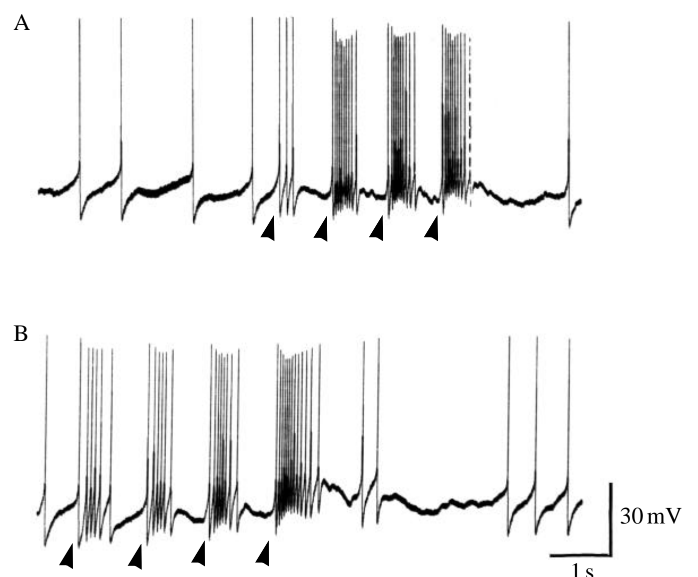

Fig. 6. Mechanical stimulation of the head (A) and tail (B) of minimally dissected animals results in spike bursts in Pd-SW neurons. Each arrowhead represents the delivery of a single mechanical stimulus to the animal.

bursting in Pd-SW neurons. Throughout the entire study, such recordings from various cell types provided over 300 recordings from which information on the potential modulation of swim frequency could be obtained. Data from recorded swim interneurons, general excitor and small motor neurons and muscle cells are presented in Fig. 7. In all recorded cell types, no significant differences in swim cycle frequency were found following induced Pd-SW cell activity. In most of these recordings, simultaneous force transducer measurements confirmed that the levels of induced activity in recorded Pd-SW cells were capable of producing the peripheral modulatory effect, increased muscle contractility. In five preparations (data not shown), various pairs of Pd-SW neurons were simultaneously stimulated while swim frequency was monitored *via* a force transducer. Some of the pairs included neurons of the pedal cluster that innervated targets other than the wings. As with stimulation of individual Pd-SW neurons, swim frequency was unchanged following simultaneous activity in mixed cell pairs.

Pattern generator interneurons

Stimulation of Pd-SW cell bursts during periods of swimming quiescence did not produce electrical responses in swim interneurons, suggesting that Pd-SW neurons do not directly affect interneuron activity (Fig. 8A). Swim interneurons produce a single broad action potential with each swim half-cycle. To test for possible modulatory effects of Pd-SW cells on swim interneuron activity, action potential durations were measured prior to and 2 s after induced Pd-SW bursts (N=16 different preparations). In these experiments, there was no change in interneuron action potential duration following Pd-SW cell stimulation (data not shown).

As noted earlier, initiation of swimming activity is accompanied by distinct excitatory synaptic inputs to Pd-SW neurons, resulting in tonic depolarization for the duration of the swimming bout. Two observations suggest that this depolarizing activity is not directly driven by swim interneuron activity. First, the barrage of synaptic inputs to Pd-SW cells often included discrete EPSPs that occurred at a much higher frequency than that of swim interneuron activity (Fig. 8A). Second, stimulation of spike activity in swim interneurons during periods of swim quiescence did not produce detectable synaptic inputs in Pd-SW cells (Fig. 8B).

Small motor neurons

Small motor neurons are active during both slow and fast swimming and are primarily responsible for motor drive during slow swimming. Stimulation of Pd-SW neurons during periods of swim quiescence, or in preparations bathed in high-Mg^{2+}, high-Ca^{2+} sea water, did not produce synaptic input to small motor neurons (Fig. 9A). Likewise, motor neuron activity had no effect on Pd-SW cell membrane potential. This is illustrated in the experiment shown in Fig. 9, which was conducted in high-Mg^{2+}, high-Ca^{2+} sea water. Since swimming was blocked by this treatment, the motor neuron had to be driven *via* intracellular depolarization (driven at 1.3 Hz). Visual observation of wing activity in this and similar experiments indicated that only a restricted region of the wing contracted during induced motor neuron activity, suggesting that other coupled neurons, most notably the general excitor motor

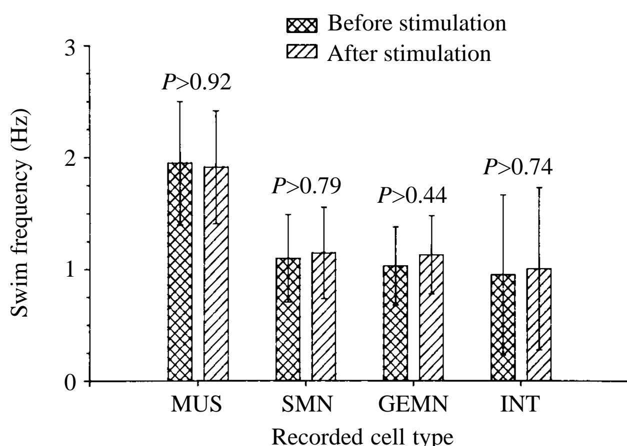

Fig. 7. Swim frequency does not change significantly following Pd-SW neuron stimulation as judged from recordings from muscle cells (MUS), small motor neurons (SMN), general excitor motor neurons (GEMN) and swim interneurons (INT) (Student's t-test). The overall frequency of muscle cell recordings is high since the sample included both slow-twitch and fast-twitch muscle cells. The latter are active only during fast swimming, which exhibits a higher swim frequency (Student's t-test). Error bars represent ± S.E.M., N>300.

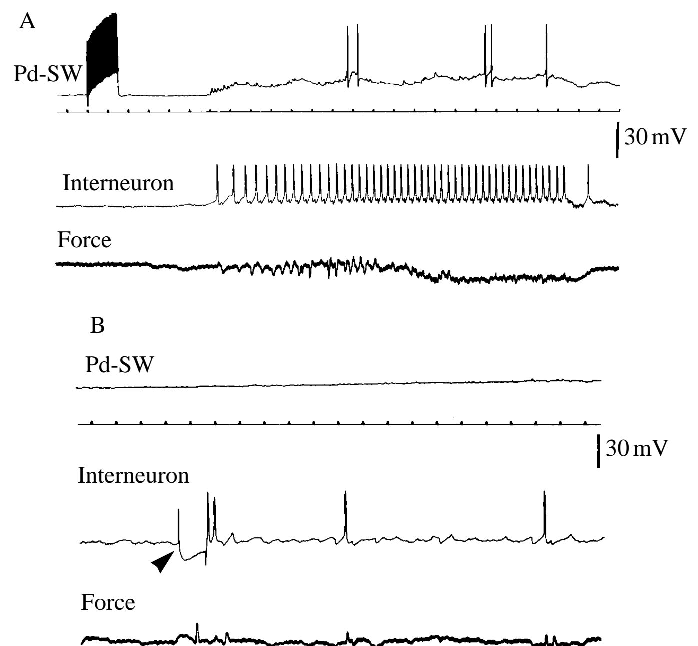

Fig. 8. (A) Activity in Pd-SW neurons does not influence swim interneuron membrane potential. Note that there is no motor response in the force trace in response to the Pd-SW neuron burst. (B) Induced or spontaneous swim interneuron spikes do not influence the membrane potential of Pd-SW neurons. The first spike doublet was induced *via* post-inhibitory rebound by hyperpolarizing the membrane at the arrowhead. The following two spikes were spontaneous. The tick interval on the time traces is 1 s.

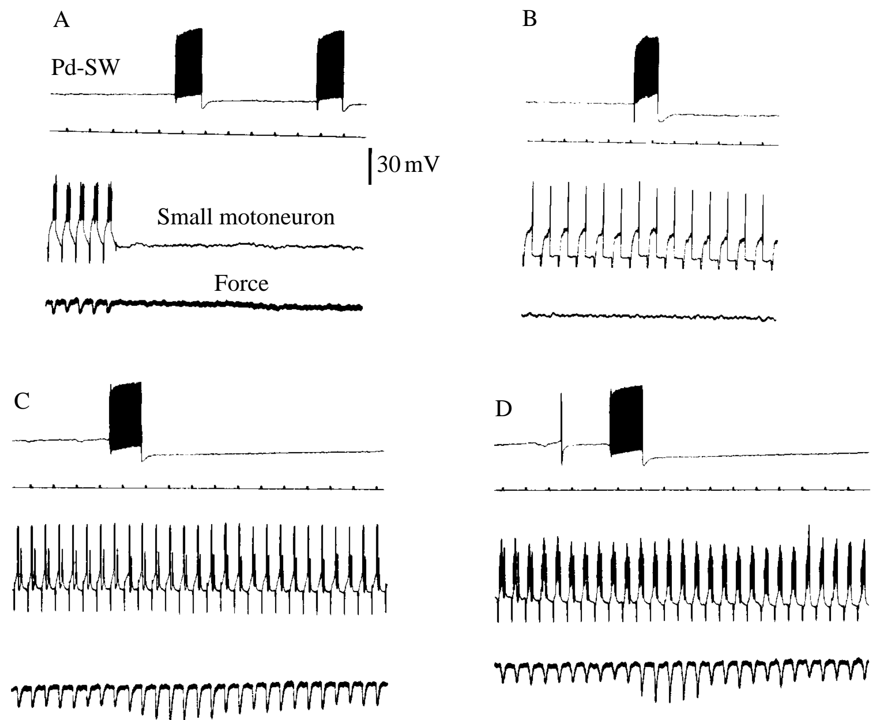

Fig. 9. Pd-SW neurons do not directly influence the activity of small motor neurons. Swimming activity was blocked by placing the preparation in high-Mg^{2+}, high-Ca^{2+} sea water. Motor neuron activity was driven by injecting repetitive currents through the recording electrode. Note that neuromuscular transmission is not blocked by the high-Mg^{2+}, high-Ca^{2+} solution. (A) Induced spike bursts in Pd-SW neurons do not alter the membrane potential of small motor neurons during periods of swim inactivity. (B) The stimulus current was adjusted just below threshold to show that a Pd-SW burst did not raise the membrane potential to exceed the threshold level. Fast responses in the motor neuron trace are stimulus artifacts. (C) The stimulus current was raised so that a single action potential (large spike) was triggered per stimulus. A Pd-SW burst did not increase the number of action potentials per stimulus. Note that the force recording shows an increase in contractility even though the motor neuron activity did not change. (D) The stimulus current was raised to produce a burst of motor neuron action potentials per stimulus. A Pd-SW burst did not change the number of action potentials per motor neuron burst (verified at higher sweep speeds) even though there was a significant modulation of contractility as noted in the force recording. The tick interval in the time traces is 1 s.

neuron and swim interneurons, were not activated by the stimulus regime. To look for subtle modulatory influences on motor neuron activity, and to minimize the chance of stimulating coupled motor neurons, the motor neuron stimulus strength was varied from just below spike threshold (Fig. 9B) to just above threshold, so that only a single spike was produced per stimulus (Fig. 9C). Then, stimulus strength was increased so that spike bursts were produced with each stimulus (Fig. 9D). When adjusted to just below threshold, even intense bursts of Pd-SW cell spikes produced no repeatable changes in motor neuron activity. The recording shown in Fig. 9B shows a further example of the lack of motor effect of Pd-SW cell activity. When motor neuron stimulus strength was adjusted to produce only a single spike per stimulus, Pd-SW cell activity did not induce an increase in motor neuron spike output, even though clear modulation of contractile strength was evident (Fig. 9C). Similarly, with the production of motor neuron spike bursts, the number, frequency or duration of action potentials within each burst was not altered by Pd-SW cell activity, although Pd-SW activity was shown to produce a distinct and repeatable effect on contraction amplitude (Fig. 10). Since these experiments were conducted in high-Mg^{2+}, high-Ca^{2+} sea water, and the resulting wing contractions were always observed to involve small, restricted regions of the ipsilateral wing, it was concluded that only single motor neurons were being stimulated. It was thus highly unlikely that the observed modulation of contractile strength was due to *central* modulatory effects on small motor neuron activity.

General excitor motor neurons

Identical results were obtained in experiments designed to screen for modulatory effects on general excitor motor neurons. Pd-SW cell activity did not produce changes in the membrane potential of general excitors, nor did general excitor activity affect Pd-SW cell membrane potential (Fig. 11A). Changes in general excitor activity following Pd-SW cell activation were not noted in cells whose activity was just below or just above threshold (Fig. 11B) or in either chemically blocked (high-Mg^{2+}, high-Ca^{2+} sea water) or spontaneously active preparations (Fig. 11C).

Peripheral effects of Pd-SW cell activity

Slow-twitch muscles

The organization of peripheral projections of the Pd-SW neurons (Satterlie *et al.* 1995) strongly suggests that a modulatory target is the slow-twitch swim musculature of the wings. Intracellular recordings from individual slow-twitch muscle cells revealed three types of responses to induced bursts in Pd-SW neurons during existing swimming activity. Ordinarily, slow-twitch muscle cells produced either a single excitatory junctional potential (EJP) or a graded spike-like response during each half-cycle of swimming activity. When muscle responses consisted of EJPs only, the most frequently recorded modulatory response involved a change from EJP activity to the production of spike-like responses (Fig. 12A). In all cases, the increased activity appeared with a latency of approximately 1 s following initiation of the Pd-SW cell burst and lasted for no more than 8–10 s, thus mirroring the duration

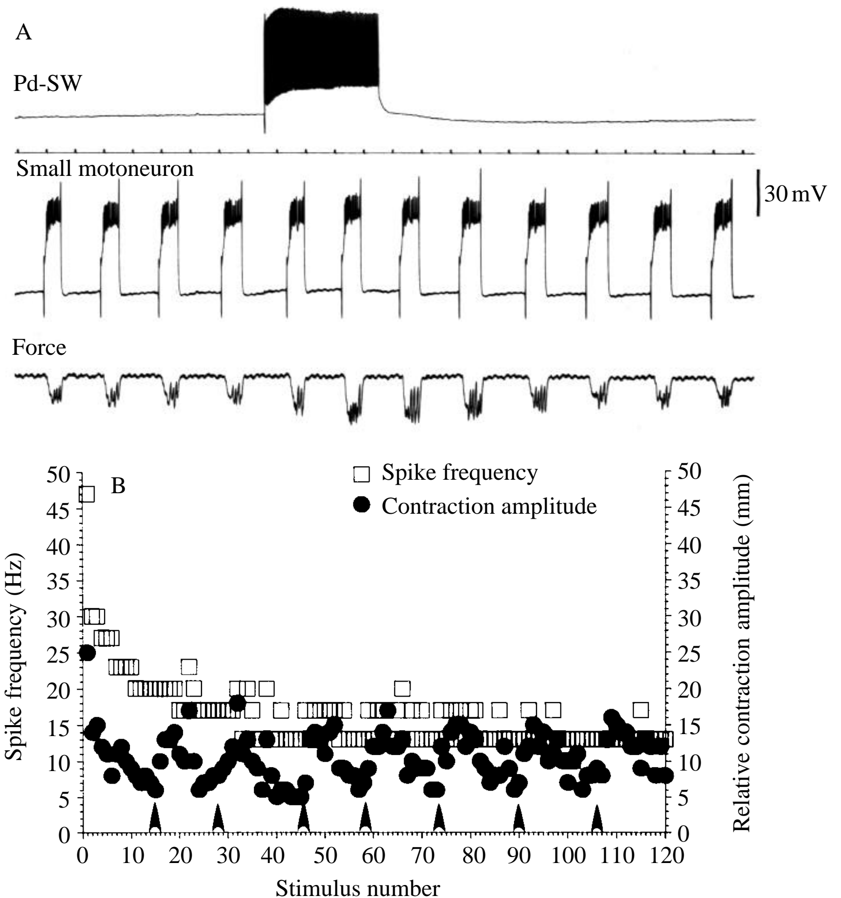

Fig. 10. The spike frequency within small motor neuron bursts does not change following Pd-SW neuron activity, while wing contraction amplitude does. The experimental procedure was similar to that used in Fig. 9D, but Pd-SW bursts were triggered every 20–30 s while the motor neuron was stimulated at 0.5 Hz (stimulus number). One such trial is shown in A. (B) The results of the entire series. At each arrowhead, a Pd-SW neuron burst was triggered. Note that the spike frequency starts high and falls to a steady-state level, whereas the contraction amplitude increases following each Pd-SW neuron burst. The tick interval in the time trace is 1 s.

of the whole-wing modulatory responses measured with the force transducer. As in the force recordings, the enhancement of electrical activity was gradual, reaching a peak 2–4 s after the initiation of the Pd-SW cell burst. In a few recordings in which EJP activity was measured, the modulatory response consisted of an enlargement of the EJPs without the production of a spike-like responses (Fig. 12B). Once again, the temporal pattern of the modulatory response was identical to that produced in whole-wing contractions. This is best seen in Fig. 12B, in which the changes in EJP activity correspond with changes in the contractile activity of the wing measured with a force transducer placed in the vicinity of the recorded muscle cell.

Finally, when the existing activity of the muscle cells involved the production of large spike-like responses, induced bursts in Pd-SW neurons produced a transient increase in the size of the spike-like responses (Fig. 12C) without a significant change in their duration. As with the other two types of modulatory responses, the time course of spike enhancement was within the range of the whole-wing responses.

Fast-twitch muscles

Similar experiments involving intracellular recordings from fast-twitch muscle cells were hampered by the absence of electrical activity or the intermittent appearance of small EJPs during slow swimming. Bouts of fast swimming, during which fast-twitch muscle typically produced spike activity, were not used since the level of spontaneous firing in Pd-SW cells was high throughout the fast swimming episodes. Direct stimulation of general excitors was ruled out for three reasons. First, electrical coupling between general excitors and some small motor neurons resulted in unwanted stimulation of the latter, even with single spikes (similar stimulation in the opposite direction, i.e. from small motor neurons to general excitors, was seldom observed). Second, our preliminary evidence suggests that there may be chemical excitatory contacts between the general excitors and some synergistic small motor neurons. Finally, the general excitors innervate both types of muscle cells. To overcome these problems, fast-twitch muscle cells were depolarized intracellularly at various frequencies during periods of swim quiescence or during slow swimming. Fig. 13A shows both subthreshold and suprathreshold responses to such intracellular depolarization. The response to the latter was either a burst of facilitating spikes when the stimulus strength was well above threshold (Fig. 13A) or a single spike followed by small membrane oscillations when the stimulus strength was just above threshold (shown only at slow sweep speed in Fig. 13C).

When fast-twitch muscle cells were depolarized with subthreshold stimuli, induced bursts of Pd-SW neuron spikes did not alter the membrane responses of fast-twitch cells (Fig. 13B). Likewise, when the stimulus strength was adjusted

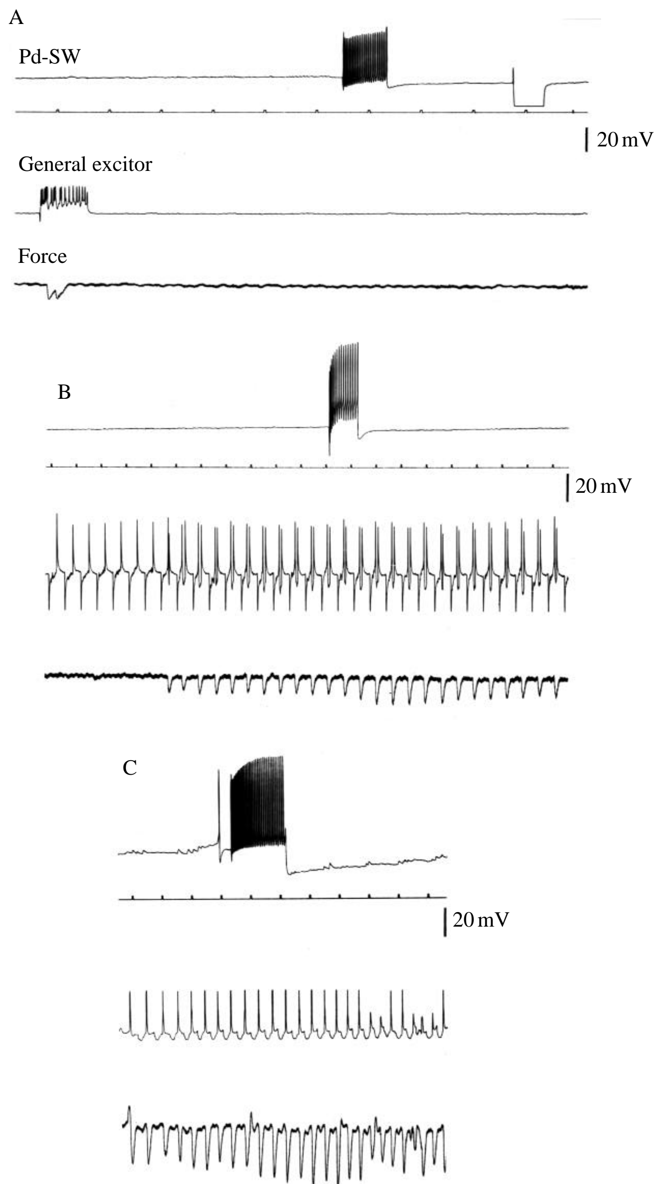

Fig. 11. Pd-SW neuron activity does not alter general excitor motor neuron activity. Recordings A and B were taken from preparations maintained in high-Mg^{2+}, high-Ca^{2+} sea water, while those shown in C were obtained from preparations in normal sea water. (A) A spike burst in the general excitor produces a contraction of the wing, while an induced burst in the Pd-SW neurons does not alter the membrane potential of the motor neuron. (B) When repetitive stimuli are delivered to the motor neuron, initially at a subthreshold level, contractions do not appear in the force trace (note the stimulus artifacts). When the stimulus intensity is then adjusted to produce a single action potential per stimulus, an induced burst in the Pd-SW neuron does not result in an increase in motor neuron spike number even though an increase in muscle contractility is recorded. (C) Stimulation of a Pd-SW neuron spike burst during spontaneous swimming activity in the motor neuron does not alter the motor neuron activity. The tick interval in the time traces is 1 s.

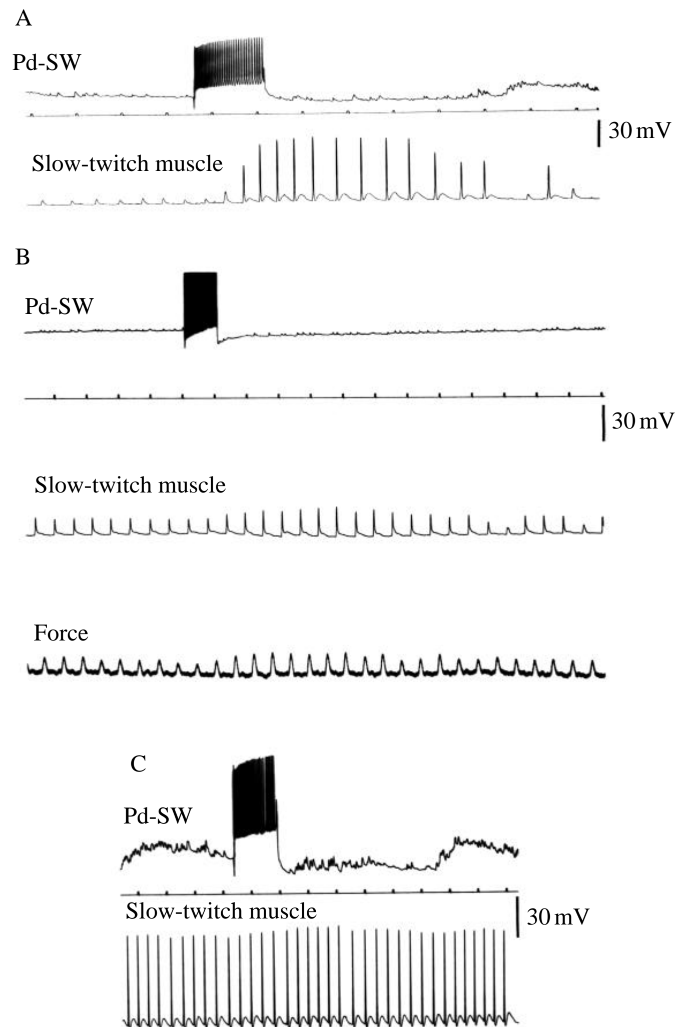

Fig. 12. Pd-SW neuron activity enhances the electrical activity of the slow-twitch swim musculature. Intracellular recordings from slow-twitch muscle cells revealed three types of effect following induced spike bursts in Pd-SW neurons. Muscle cells that were producing a single junctional potential with each swim muscle contraction either began producing spike-like potentials (A) or enlarged junctional potentials (B). Note that the enhancement of junctional potentials in B follows a time course similar to that of the enhancement of contractility in the force recording. When muscle cells were producing near maximal spike-like potentials, a Pd-SW neuron burst triggered a transient increase in the amplitude of the spike-like responses (C). In A and C, the Pd-SW cells were hyperpolarized slightly to prevent spontaneous spiking prior to and after the induced burst. The tick interval on the time traces is 1 s.

to just above threshold, the size of the muscle spike-like responses did not change, as observed in slow-twitch cells (Fig. 13C). Repeating the experiment at different muscle cell stimulus frequencies (within the range of normal swimming frequencies) did not alter the results.

Is serotonin the modulatory agent?

To test the role of serotonin further in the production of peripheral modulatory responses, preparations in which whole-wing contractile force was monitored while the Pd-SW neurons were stimulated were used in conjunction with the serotonin antagonist mianserin. As shown in Fig. 14, the typical force modulation observed following a spike burst in the Pd-SW

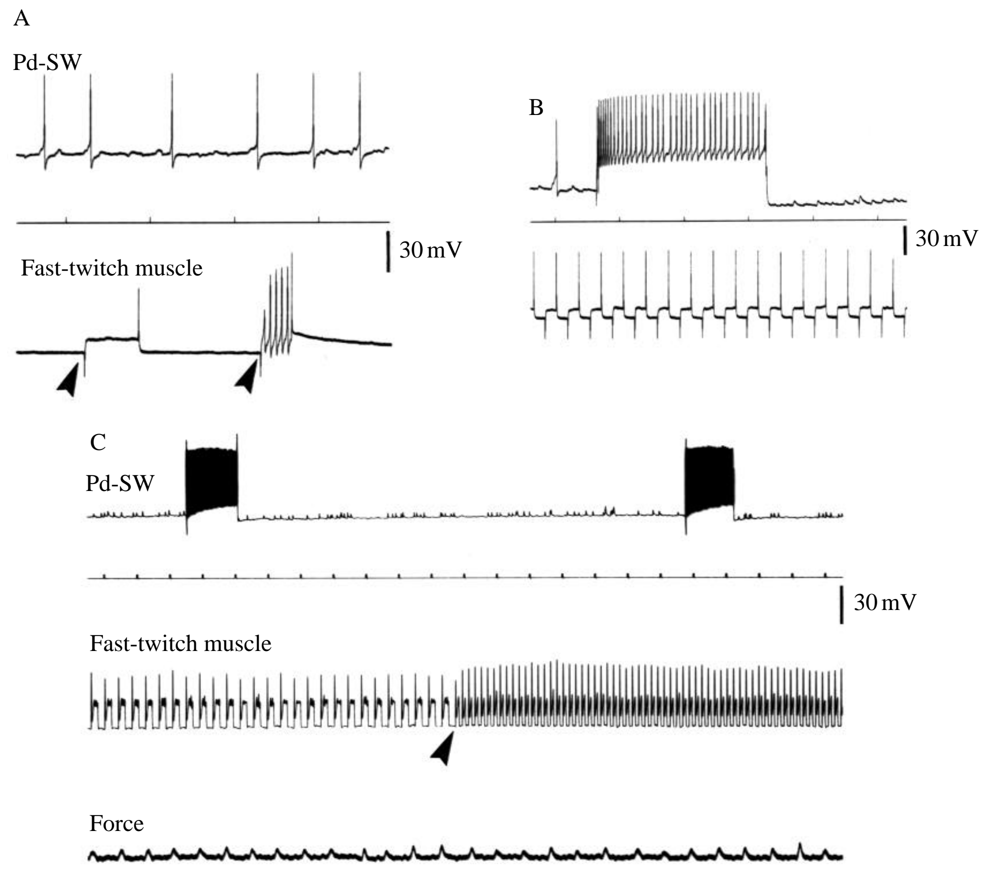

Fig. 13. Pd-SW neuron activity does not alter the electrical activity of fast-twitch swim musculature. (A) A subthreshold stimulus (first arrowhead) and a stimulus well above threshold (second arrowhead) were delivered to show that above threshold the fast-twitch muscle cell responded with a burst of spike-like responses. At just above threshold, the muscle cells responded with an initial spike-like response followed by membrane oscillation (shown at slow sweep speed only in C). (B) The muscle stimulus amplitude was adjusted to just below threshold and a Pd-SW neuron was stimulated to produce a spike burst. There was no apparent enhancement of the subthreshold voltage responses of the muscle cell following the Pd-SW burst. (C) The muscle stimulus current was adjusted to just above threshold. Bursts in Pd-SW neurons did not trigger an alteration in the size of spike-like responses in the muscle cell. Stimuli were delivered at two frequencies (3 Hz and 6 Hz) that corresponded with a normal range for fast swimming. Note that the frequency of the induced activity in the muscle cell did not correspond with the frequency of existing swimming activity (force recording). The tick interval of the time traces is 1 s.

neuron (Fig. 14A) was blocked following 10 min of mianserin superfusion (Fig. 14B). The modulatory responses reappeared after 15–30 min of washing in sea water (Fig. 14C).

Discussion

The electrophysiological data support the morphological evidence suggesting that the medial cluster of pedal serotonin-immunoreactive (Pd-SW) neurons is involved in the modulation of swimming activity. While no direct motor effect on swim musculature is produced by activity in these cells, it is capable of temporarily increasing muscle contractility in swimming preparations. Modulatory responses were only noted in slow-twitch muscle cells, where Pd-SW neuron activity increased the size of muscle cell EJPs, increased the size of spike-like responses or changed EJP activity to spiking activity. Similar increases in the size of muscle responses to serotonin or serotonin-releasing neurons have been noted in molluscs, both with (e.g. Weiss *et al.* 1978; Ram *et al.* 1981; Zoran *et al.* 1989) and without increases in muscle relaxation rate (e.g. Dorsett *et al.* 1989; Lloyd, 1980; McPherson and Blankenship, 1991, 1992). There was no significant change in the relaxation rate of muscle contractions in *Clione limacina* muscles following activity in Pd-SW neurons. Such changes would not be required in this case since (1) modulation of muscle activity was not accompanied by an increase in swim frequency, and (2) muscle twitches were produced by rapid spike-like responses and thus were relatively fast events, frequently occupying a small proportion of the cycle period. The primary change in contraction kinetics occurred when a muscle cell changed from the production of EJPs to the production of spike-like responses, as the rise time of the contractions appeared to mirror the difference in the rate of voltage change.

The action of the Pd-SW neurons appears to be purely peripheral because increases in swim frequency or motor neuron activity were not found. In addition, no change was noted in the duration of interneuron or motor neuron action potentials. Peripheral modulation was observed following Pd-SW neuron activity even when single action potentials were triggered in individual motor neurons, indicating that the increased force was not due to an increase in motor neuron burst activity. Furthermore, during spontaneous or induced motor neuron activity consisting of bursts of spikes, increases in spike number did not accompany peripheral modulation of contractility.

Serotonin immunoreactivity, on its own, does not indicate an active role for serotonin in producing the peripheral modulatory responses, particularly since many molluscan neurons have been shown to contain and release multiple transmitters/modulators (e.g. Church and Lloyd, 1991; Cropper *et al.* 1987*a*,*b*, 1990; Whim and Lloyd, 1989). The

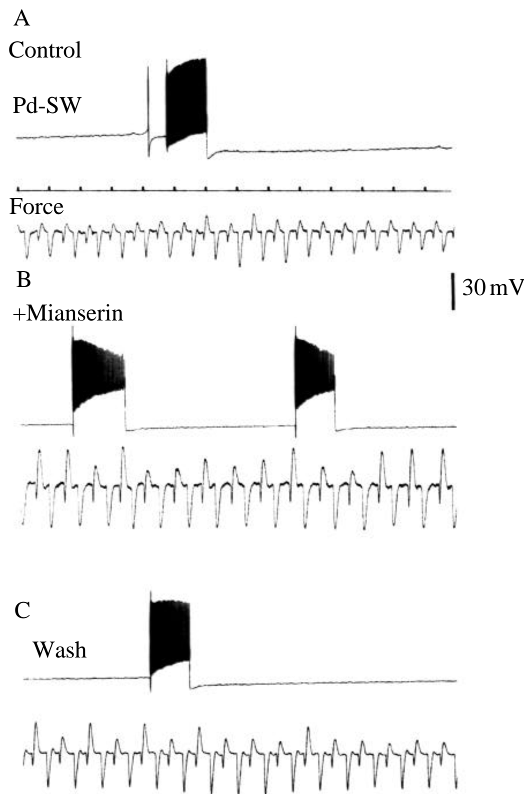

Fig. 14. The enhancement of contractility induced by Pd-SW neuron activity (A) is reversibly blocked by the serotonin antagonist mianserin (10 μmol l^{-1}; B and C). The tick interval on the time trace is 1 s.

reversible blockade of modulatory responses by the serotonin antagonist mianserin strongly suggests that serotonin performs a primary role in peripheral modulation.

The restriction of the significant modulatory effects of Pd-SW neurons to the swim musculature suggests that the serotonergic system involved in swim modulation includes separate central and peripheral components. In this regard, the Pd-SW neurons serve a similar function to that of the serotonergic POP cells of *Aplysia* species (McPherson and Blankenship, 1991, 1992). As with *Clione limacina* neurons, interconnections between POP neurons of *Aplysia brasiliana* were not found. The POP neurons were normally quiescent, but became rhythmic bursters in the presence of bath-applied serotonin (Parsons and Pinsker, 1989). Owing to the lack of interconnections, POP neurons fired asynchronously under these conditions, but were entrained when the swim motor program was initiated, thus suggesting common inputs to individual POP neurons. Similarly, the Pd-SW neurons of *Clione limacina* do not show electrical or chemical synaptic coupling, but they do receive common inputs from unknown sources, and all increase their firing rates during swimming activity. In contrast to the POP cells of *A. brasiliana*, the Pd-SW cells of *Clione limacina* do not fire in phase with swimming activity, but instead produce irregular spike activity that changes frequency in parallel with changes in the frequency of swimming movements. Similar results were obtained by Sakharov and Kabotyansky (1986) and Sakharov (1990), who undoubtedly recorded from the same group of neurons, which they identified on the basis of their histofluorescence preparations. Furthermore, POP neurons were found to have peripheral axons that ran to the ipsilateral parapodium and to produce enhancement of motor-neuron-driven parapodial contractions. The POP neurons of *A. brasiliana* thus appear to be functionally similar to the Pd-SW neurons of *Clione limacina*.

Since the Pd-SW neurons of *Clione limacina* are the only serotonin-immunoreactive cells thus far found to innervate the swim musculature and the number of immunoreactive axons in the wing nerves matches the number of cells innervating the periphery from this cluster (Satterlie *et al.* 1995), it is likely that the serotonergic peripheral modulatory component is restricted to the Pd-SW cell cluster. Cerebral serotonin-immunoreactive neurons that produce central modulation of swimming activity have also been identified in *Clione limacina* and appear to have no direct peripheral affects (Satterlie and Norekian, 1995). A similar separation of central and peripheral modulation has been found in the leech heartbeat system (Calabrese and Arbas, 1985).

One interesting feature of the Pd-SW neurons of the *Clione limacina* swimming system is that they only innervate the slow-twitch muscles, as substantiated by immuno-histochemistry, cell injections and electrophysiological data. The selective innervation is curious, particularly because fast-twitch muscles are recruited during the change to fast swimming. The relatively fatigue-resistant properties of the two muscle groups, however, suggest that modulation is biased towards the fatigue-resistant (more 'stable') slow-twitch fibers rather than the fast-twitch fibers, which show significant fatigue during repetitive activity (Satterlie *et al.* 1990). The fast-twitch fibers will thus have their greatest effect immediately after recruitment in the initial period of swim acceleration and will be 'backed up' by enhanced contractility of the slow-twitch fibers if modulated by the Pd-SW neurons.

What is the role of peripheral modulation in swimming speed changes? A 'change of gears' from slow to fast swimming involves central changes in the interneuron and motor neuron systems, including acceleration of the swim frequency, addition of interneuron types to the pattern generator, recruitment of the general excitor motor neurons and an increase in burst activity in both motor neuron types. The Pd-SW neurons produce none of these responses. They do, however, induce an increase in contractility of the swim musculature, which could translate into a subtle change of speed *within gears*. If the compartmentalized serotonin modulatory system were considered to act in a yes/no manner, the following four possible swimming states could be obtained. (1) No modulation – slow swimming, normal muscle contractility. (2) Central modulation only – fast swimming, normal muscle contractility (normal for fast swimming). (3) Peripheral modulation only – slow swimming, enhanced muscle contractility. (4) Both central and peripheral modulation – fast swimming, enhanced muscle contractility.

With this scheme, the change from 1 to 3 could involve a change of speed within the slow 'gear', while a change from

2 to 4 would represent the same for fast swimming. Biomechanical evidence of a role for increased muscle contractility in the absence of cycle frequency changes is not available. Likewise, synaptic interactions between neurons that produce acceleration of swim frequency (Satterlie and Norenkian, 1995) and Pd-SW neurons suggest that the two groups typically act together. Despite the lack of direct evidence of independent activity in Pd-SW neurons, the separation between the central and peripheral serotonergic modulatory subsystems suggests that such independent activity could play a role in altering locomotory performance.

I thank Dr A. O. D. Willows, Director of Friday Harbor Laboratories, for providing space and generous assistance, Lou and Alison Satterlie and Dr Claudia Mills for help in collecting animals and Dr T. Norekian and S. Jordan for critically reading the manuscript. The study was supported by NIH grant R01 NS27951.

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