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Differential channeling of sensory stimuli onto a motor neuron in the leech

Abstract We studied a specific sensory-motor pathway in the isolated leech ganglia. Pressure-sensitive mechanosensory neurons were stimulated with trains of action potentials at 5–20 Hz while recording the responses of the annulus erector motorneurons that control annuli erection. The response of the annulus erector neurons was a succession of excitatory postsynaptic potentials followed by inhibitory postsynaptic potentials. The excitatory postsynaptic potentials had a brief time-course while the inhibitory postsynaptic potentials had a prolonged time-course that enabled their temporal summation. Thus, the net effect of pressure-sensitive neuron stimulation on the annulus erector neurons was inhibitory. Both phases of the response were mediated by chemical transmission; the excitatory postsynaptic potentials were transmitted via a monosynaptic pathway, and the inhibitory postsynaptic potentials via a polysynaptic one. The pattern of expression of this dual response depended on the field of innervation of the sensory neuron and it was under the influence of cell 151, a non-spiking interneuron, that could regulate the expression of the hyperpolarization. The interaction between pressure-sensitive neurons and annulus erector neuron reveals how sensory specificity, connectivity pattern and regulatory elements interplay in a specific sensory-motor network.

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Abbreviations *AE* annulus erector · *EPSP* excitatory postsynaptic potential · *IPSP* inhibitory postsynaptic potential · *P-cell* pressure-sensitive neuron

Introduction

The appropriate channeling of sensory signals through motor pathways shapes the behavioral responses of an animal to external stimuli. Therefore, it is of interest to understand the mechanisms through which sensory information is conveyed onto motorneurons (Grillner et al. 1991; Burrows 1992; Morton and Chiel 1994). Invertebrates, due to the relatively simple structure of their organisms, are good models to undertake detailed cellular studies on sensory-motor integration. The medicinal leech, for example, is composed of a series of highly similar segments, each of which shows relative sensory and motor autonomy. Each segment is innervated by a ganglion, where specific sensory and motor neurons can be readily identified by their soma location and their electrophysiological properties (Muller et al. 1981). Detailed studies of the neuronal networks underlying a specific motor response, the local bending reflex, showed that sensory-motor integration in the leech can follow a complex distributed network, in which the final motor output is shaped by processing a specific sensory signal through multiple parallel pathways acting in conjunction (Lockery and Kristan 1990; Lewis and Kristan 1998).

The aim of the current study was to characterize the complex interaction between a pair of specific sensory and motor neurons. The annulus erector (AE) cells are a pair of motorneurons present in each segmental ganglion that innervate the muscles responsible for erecting the annuli into sharp ridges. Their innervation field extends from the dorsal to the ventral midline of the side contralateral to the location of the

soma, covering the central annulus of the segment plus four annuli on either side of it. The firing rate of AE neurons determines the degree of contraction of the muscles erecting the annuli, producing a marked change in the indentation of the skin (Stuart 1970). AE motorneurons respond to several sensory stimuli: diffuse illumination onto the body wall, stimulating the sensillar photoreceptors, evokes a burst of excitatory potentials in the AE motorneurons (Kretz et al. 1976); water movements, stimulating the sensillar movements receptors, lead to their inhibition (Friesen 1981); and stimulation of specific mechanosensory pressure-sensitive neurons, the P cells, produces an excitatory input onto them (Jansen et al. 1974; Gu et al. 1991). The present study extends the investigation on the P-AE pathway, showing that these sensory neurons channeled a dual synaptic signal to the AE motorneurons. Stimulation of P-cells produced a depolarizing and a hyperpolarizing response that were transmitted through parallel mono- and polysynaptic pathways, respectively. The pattern of expression of this dual response depended on the field of innervation of the sensory neuron and it was under the influence of a non-spiking interneuron that could regulate the expression of the hyperpolarization. The P-AE interaction reveals how sensory specificity, connectivity pattern and regulatory elements interplay in a specific sensory-motor network.

Materials and methods

Biological preparation

Hirudo medicinalis Linnaeus, weighing 2–5 g, were obtained from commercial supplier (Leeches USA, Westbury N.Y.) and maintained at 15 °C in artificial pond water. The animals were not fed for at least 1 month prior to dissection. Single ganglia were dissected out of the animal and pinned ventral side up in a recording chamber lined with Sylgard (Dow Corning). In the experiments described in Fig. 2, the ganglia were superfused with various saline solutions.

Solutions

The ganglia were bathed in standard saline solution with the following composition (in mmol l^{-1}): NaCl 115; KCl 4; $CaCl_2$ 1.8; $MgCl_2$ 1; TRIS maleate 4.6; TRIS base 5.4; and glucose 10; pH 7.4. To block synaptic transmission we used a solution containing 20 mmol l^{-1} $MgCl_2$ and 1 mmol l^{-1} $CaCl_2$ (Baylor and Nicholls 1969). To discriminate between mono- and polysynaptic connections between neurons we used a solution containing 10 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} $CaCl_2$ (Jansen et al. 1974). The osmolarity was kept constant by reducing the NaCl concentration.

Electrophysiological recordings

Intracellular recordings were made with glass microelectrodes filled with a 3 mol l^{-1} potassium acetate solution, which had a 15- to 30-MΩ resistance, connected to an amplifier operating in the current clamp configuration (Axoclamp 2A, Axon Instrument, Foster City, Calif.). The neurons P, AE and 151 were recognized by their soma location and their electrophysiological properties (Nicholls and Baylor 1968; Stuart 1970; Wadepuhl 1989, respectively). P-cells were stimulated by trains of suprathreshold step pulses (2–4 nA, 5 ms), delivered by a stimulator and triggered by the acquisition software (Clampex, Axon Instrument). The voltage recordings were digitized using a TL-1 DMA interface (Axon Instruments) and acquired at a frequency of 1 kHz.

Notation

P_d and P_v correspond to P cells innervating the dorsal and the ventral skin, respectively, and AE_c and AE_i correspond to AE neurons contralateral and ipsilateral, respectively, to the stimulated P-cell.

Results

Response of AE motorneurons to P cell stimulation

Stimulation of P-cells with a train of five action potentials at 5 Hz produced a series of inhibitory postsynaptic potentials (IPSP) in the AE neurons preceded by small and phasic excitatory postsynaptic potential (EPSP) (Fig. 1A). The EPSPs and the IPSPs followed one to one each action potential in P-cells, but we frequently observed that the first action potential failed to produce a detectable response. To improve the visualization of the EPSPs we hyperpolarized the AE neuron to −70 mV, increasing the driving force of the depolarization and diminishing the driving force of the inhibition (Fig. 1A). The EPSPs had a brief time-course, with an average latency – measured from the peak of the action potential to the EPSP peak – of 18 ± 1 ms ($n = 46$). The IPSPs had a more prolonged time-course with an average latency of 39 ± 1 ms. Stimulation of P cells with a train of five action potentials at 20 Hz produced a qualitatively similar effect on the AE neuron, but this higher frequency caused the temporal summation of the IPSPs (Fig. 1B). This produced a more effective inhibition of the spontaneous firing of the AE neuron. As evidenced at −70 mV the EPSPs showed poor temporal summation. Matching the peak of the EPSPs at −70 mV with the complex response observed at around −40 mV helped to distinguish the presence of the EPSPs in these recordings that otherwise were masked by the prominent hyperpolarizing phase (inset of Fig. 1B).

Synaptic connectivity of P and the AE neurons

To examine whether the responses of the AE neurons to P-cell stimulation were produced by chemical or electrical connections we tested their interaction in a solution containing a $[Mg^{2+}]/[Ca^{2+}]$ ratio of 20/1, that is sufficient to block chemically mediated interactions in the leech (Baylor and Nicholls 1969). The two phases of the AE responses were completely abolished by this divalent cation ratio ($n = 6$), and the effect was readily reversible after washing out with standard solution (Fig. 2A).

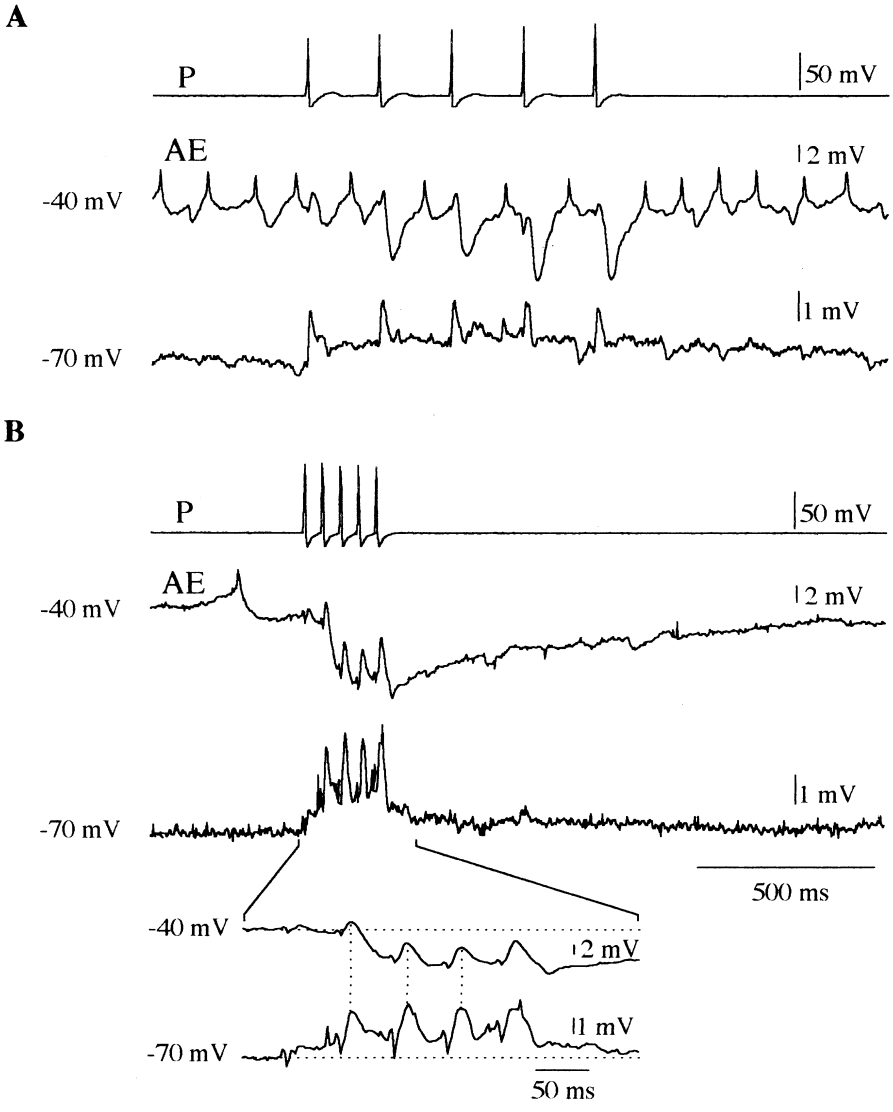

Fig. 1A, B Responses of an annulus erector (AE) neuron to pressure-sensitive (P) cell stimulation. **A** A P_d cell was stimulated by the injection of a train of suprathreshold pulses at 5 Hz and the responses of an AE neuron were studied as its membrane potential was set at around −40 mV and −70 mV, as indicated above each trace. AE neurons fired action potentials spontaneously, that are reflected as small spikes in the somatic recordings due to passive conduction from the distal active zones (Stuart 1970). **B** Similar as in **A** but the P_d cell was stimulated at 20 Hz. The *inset* at the bottom of the figure shows an amplification of a segment of the two AE recordings, corresponding to the period indicated by the lines. The *vertical dotted lines* denote the temporal matching of the peaks of the excitatory postsynaptic potentials (EPSPs) at the two membrane potentials

To examine whether the chemically mediated responses between P and AE neurons were carried through mono- or polysynaptic pathways we performed experiments in a solution containing 10 mmol l^{-1} Mg^{2+} and 10 mmol l^{-1} Ca^{2+}, that impairs polysynaptic pathways in the leech (Jansen et al. 1974). While the hyperpolarization amplitude was practically abolished in this condition (18 ±8% of control, $n = 12$), the EPSP amplitude was unaffected (110 ± 3% of control, $n = 7$) (Fig. 2B).

Differential effect of the different P-cells

Each ganglion has two bilateral pairs of P-cells: one pair innervates the dorsal quadrants (P_d) and the other pair the ventral quadrants (P_v) of the body wall. To analyze how these different P-cells affected the AE neurons we measured the amplitude of the EPSPs and the IPSPs and plotted the results in terms of the four possible pairs: P_d and P_v cells ipsilateral and contralateral to AE neurons (Fig. 3). The depolarizing response was larger ($P < 0.05$, Student's *t*-test) when elicited by P_d cells, than by P_v stimulation. We frequently observed that P_v cells failed to produce EPSPs, even when the AE neurons were held at around −70 mV. P_d cells produced a significantly larger depolarization ($P < 0.05$, Student's *t*-test) in the ipsilateral motorneurons than in the contralateral (Fig. 3A). The hyperpolarization was significantly larger ($P < 0.05$, Student's t-test) when elicited by contralateral P-cells, irrespective of their type (Fig. 3B).

Involvement of cell 151 in the P-AE circuitry

Cells 151 are a bilateral pair of non-spiking neurons present in each segmental ganglion. They are electrically coupled to most motorneurons in the leech by a rectifying junction that allows the passage of hyperpolarizing currents from them to the motorneurons (Wadepuhl 1989). To evaluate the coupling between 151 and AE cells, currents of different amplitude were injected into the soma of 151 cells as the responses of

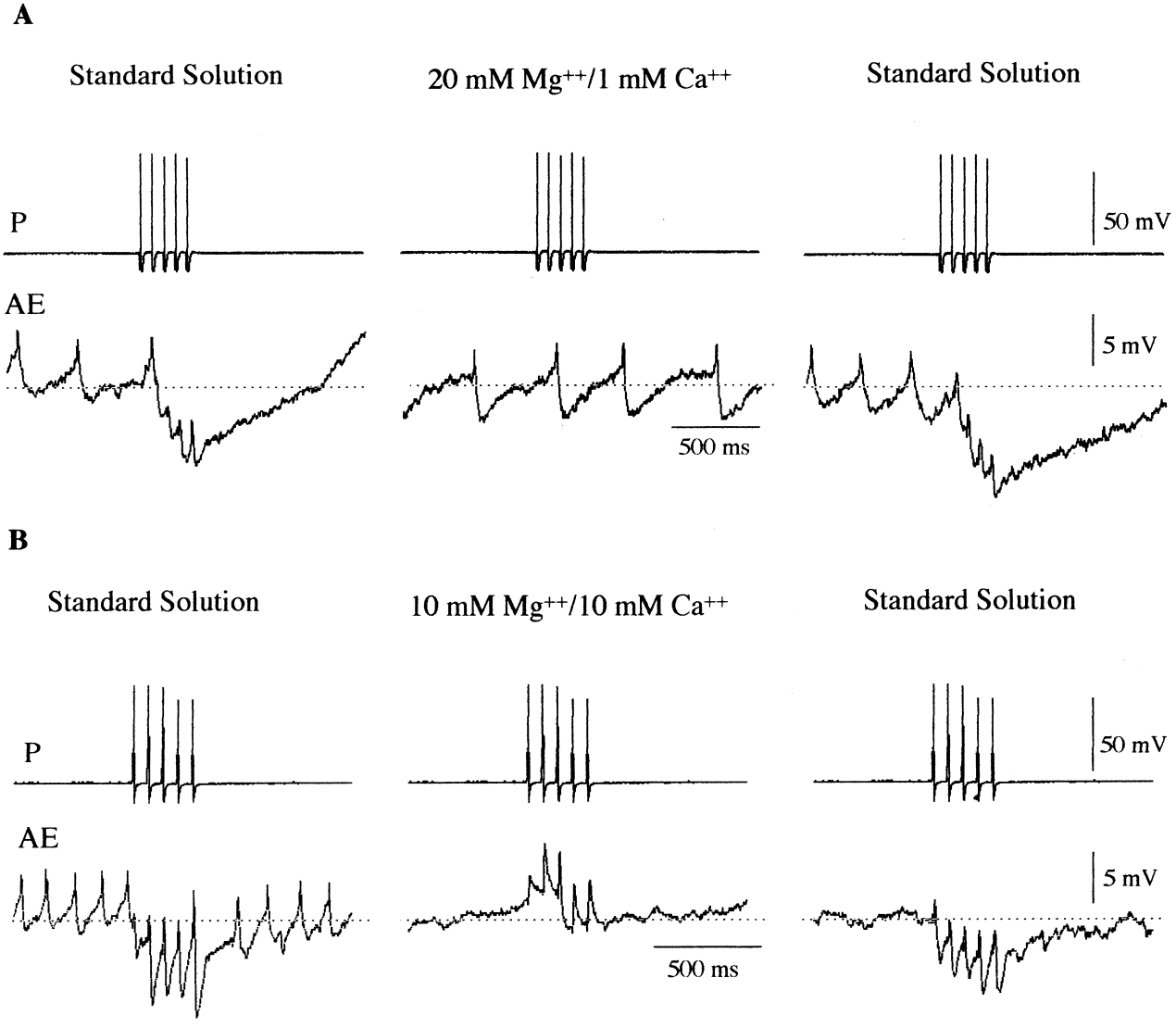

Fig. 2A, B Responses of an AE neuron in saline solutions with different Ca^{2+} and Mg^{2+} levels. The responses of an AE neuron to P cell stimulation were studied successively in standard solution, after 3 min superfusion with the test solution and after 7 min wash-out in standard solution. In **A** the test solution contained a $[Mg^{2+}]/[Ca^{2+}]$ ratio of 20/1 and in **B** the test solution contained a $[Mg^{2+}]/[Ca^{2+}]$ ratio of 10/10

AE neurons were recorded (Fig. 4A). The firing frequency of the motorneuron decreased gradually as negative current of increasing amplitude was injected, and this effect was accompanied by a gradual hyperpolarization (Fig. 4B,C). Positive currents produced a small depolarization but they did not cause any significant change in the firing frequency of the AE neurons. Injection of −7 nA into cell 151 produced a 50% reduction in AE firing frequency but a depolarizing current of the same amplitude failed to produce any measurable effect.

In a series of three microelectrodes experiments we recorded, simultaneously, the responses of an AE motorneuron and a 151 cell to P-cell stimulation. We observed that a train of five action potentials at 20 Hz in the P-cells produced a net hyperpolarization of the AE neurons and a net depolarization of cells 151 (Fig. 5A). Injecting hyperpolarizing d.c. current into cell 151 strongly diminished the hyperpolarizing phase of the AE neurons (Fig. 5B), to an extent that depended on the amplitude of the current injected into cell 151 (Fig. 5C). The EPSPs were not affected; after the injection of −12 nA their amplitude was 95.5 ± 0.03% the control value ($n = 3$).

To examine how current injection affected cell 151 response to P-cell stimulation, we compared their time integral before and during current injection. The time integral was measured as the area under the curve for a period of 800 ms from the onset of the stimulus. No difference was observed between the time integral during the injection of −12 nA with that of control (100 ± 15%). These results agree with the observation that the cell 151 response to P-cell stimulation showed a strong rectification as the cell was hyperpolarized (M. Burgin, personal communication).

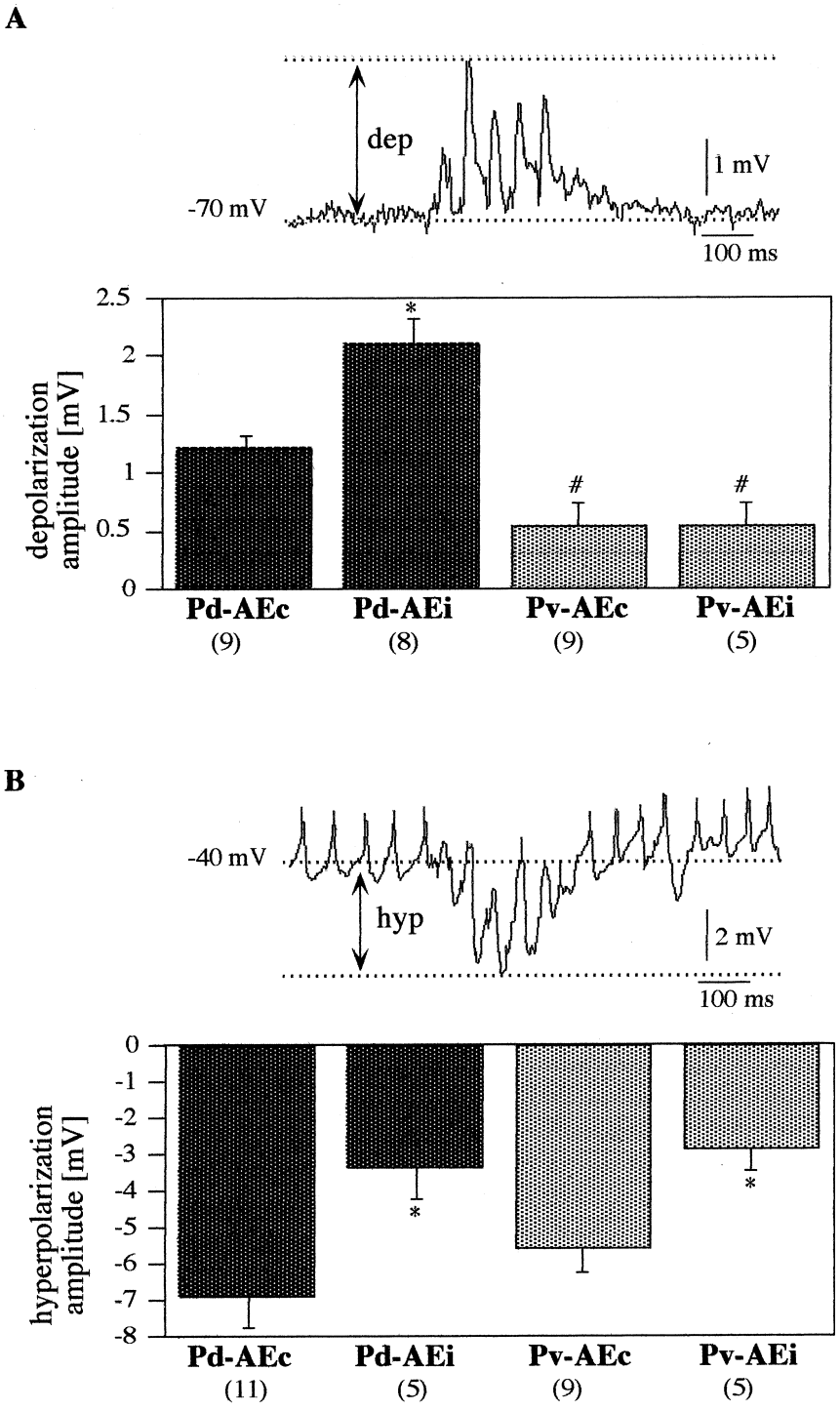

Fig. 3A, B Quantitative analysis of the AE responses. Amplitude of the responses of AE neurons contralateral (AE_c) and ipsilateral (AE_i) to P_d (*darker columns*) or P_v neurons that were stimulated by five action potentials at 20 Hz. The recordings above each plot show the way in which the amplitude of the depolarizing (**A**) and hyperpolarizing (**B**) responses were measured, as indicated by the *arrows*. The amplitude of the depolarization was measured holding AE membrane potential at around −70 mV. *Columns* indicate the average amplitude of the responses of each pair. The *bars* indicate SEM and the number in brackets indicate *n*. Symbols * and # indicated significant differences ($P < 0.05$): * compares P-AE_i with P-AE_c (lateral comparison) and # compares P_v-AE with P_d-AE (dorsal versus ventral comparison)

Discussion

The response

The results showed that AE motorneurons received a dual signal from the P-cells producing, successively, an excitatory and an inhibitory response. The EPSPs were phasic and decayed rapidly, while the IPSPs had a slower time-course. The net response of AE neurons to a train of action potentials in the P-cells, as expressed in somatic recordings, was inhibitory.

Our results differ from previous reports (Jansen et al. 1974; Gu et al. 1991) in which stimulation of P-cells with a single action potential produced the phasic depolarization, but no hyperpolarization. Those experiments were performed in high Ca^{2+} concentrations, and this may account for the absence of the hyperpolarizing

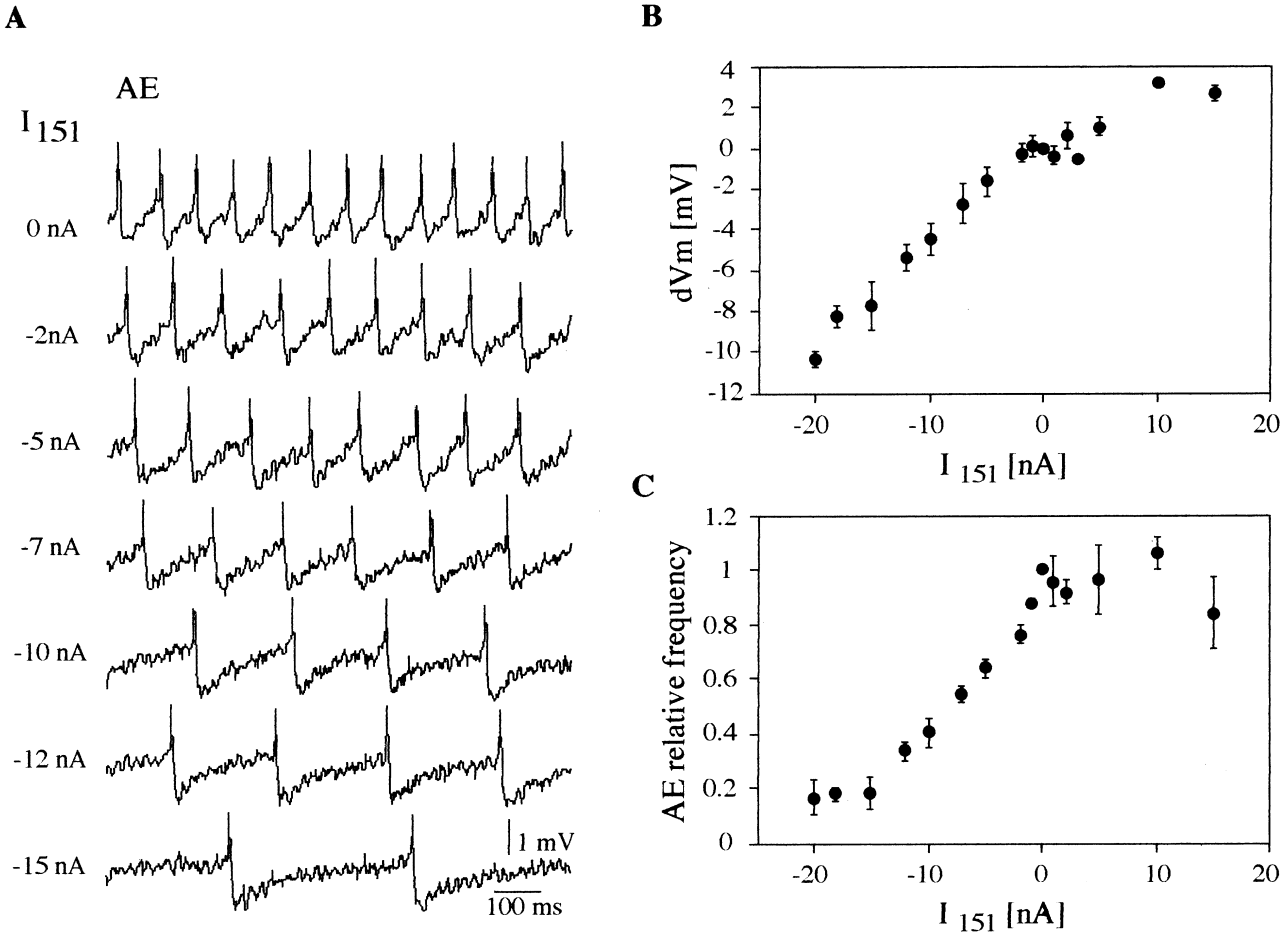

Fig. 4A–C Effect of current injection into cell 151 on AE motorneurons. **A** Traces show intracellular recordings of an AE motorneuron during the injection of hyperpolarizing currents of different amplitudes into cell 151 (I_{151}). **B** Relationship between the membrane potential changes of AE motorneurons (dVm) and the current injected into cell 151. Values are expressed as the difference between the control membrane potential ($I_{151} = 0$) and the value reached 1 s after the current injection was initiated. **C** Relationship between the change in spike frequency of AE motorneurons and the current injected into cell 151. Values are expressed as the ratio between the frequency reached 1 s after current injection was initiated and the control frequency ($I_{151} = 0$). Currents of different amplitudes were injected in random order

phase since, as we showed, the hyperpolarization failed in the presence of high cation concentrations. On the other hand, the authors did not report the membrane potential of the AE neuron at the time of the stimulus. Since their AE recordings do not show spontaneous firing, it is possible that their recordings were obtained while holding the membrane potentials at a negative value, near the reversal potential for the IPSPs.

Because of their kinetic properties, the two responses showed different temporal integration. The IPSPs showed a marked temporal summation as the stimulation frequency was increased. This caused a prolonged inhibition of the spontaneous firing of the motorneuron that, in many cases, extended beyond the stimulation period. On the other hand, the small EPSPs showed no significant temporal summation and remained in all conditions a subthreshold signal.

The connectivity pattern of the P-AE pathway

Figure 6 describes the network that results from our interpretation of the data. The monosynaptic interaction between the P and the AE neurons conveyed a depolarizing signal; this interaction was stronger for P_d than for P_v cells (Fig. 3). The hyperpolarizing signal was mediated by interneuron(s) and our results suggest they are spiking neurons, since the AE response failed in the presence of high concentrations of divalent cations (Fig. 2), a condition that raises the firing threshold (Jansen et al. 1974). Both types of P cells produced IPSPs of similar characteristics.

Cell 151 is a non-spiking neuron that is electrically coupled to most motorneurons including the AE (Wadepuhl, 1989). As a result of this interaction AE motorneuron was gradually inhibited by the hyperpolarization of cell 151 (Fig. 4). The magnitude of the current needed to exert the reported effects was similar to that applied by other authors working with non-spiking neurons (Burrows and Siegler 1978; Wadepuhl

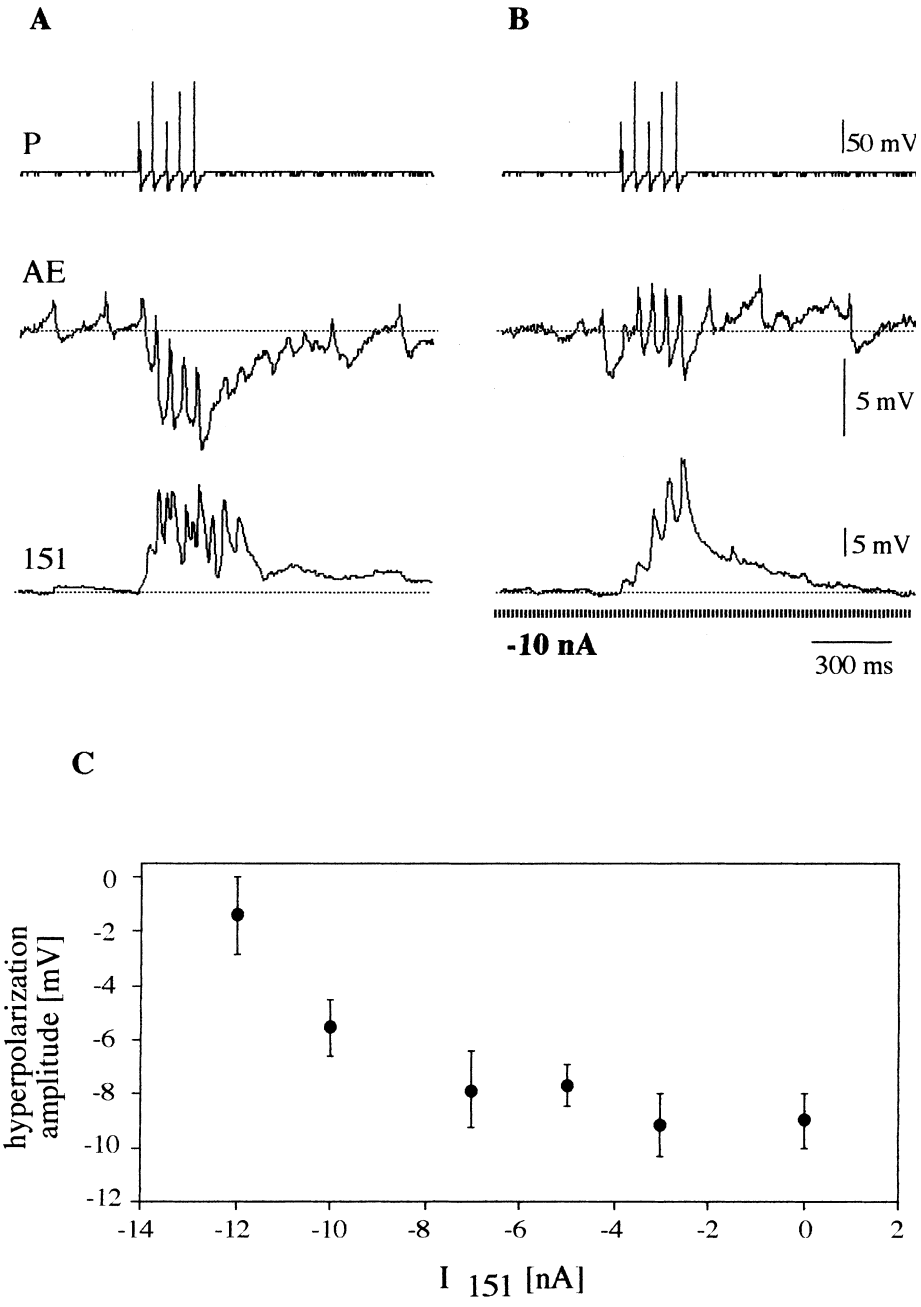

Fig. 5A–C Response of an AE neuron to P cell stimulation as cell 151 was hyperpolarized. **A** The activity of an AE neuron and a cell 151 were simultaneously recorded as a train of five action potentials at 15 Hz was evoked in a P cell. **B** The same experiment was performed while injecting a constant −10-nA d.c. current into cell 151. Since hyperpolarization of cell 151 caused the hyperpolarization of the AE neuron we injected the necessary d.c. current in the motorneuron to return its membrane potential to the control value. **C** The graph shows the relationship between the average maximal amplitude of the hyperpolarizing responses of AE neurons (see Fig. 3) and the current injected in 151 cells ($n = 3$). The bars indicate SEM. Currents of different amplitudes were injected in random order

1989). This type of neurons has extensive arborization and current injected into their soma might spread passively throughout it before reaching the sites of interaction with other neurons. Besides its direct interaction with the AE neurons, cell 151 appeared to be involved in the P-AE circuitry since its hyperpolarization uncoupled the polysynaptic phase of the response (Fig. 5). We find that the most plausible interpretation of the results is that cell 151 is also electrically coupled to the spiking interneuron(s) mediating the P-AE pathway. Due to this interaction, hyperpolarization of cell 151 could affect the firing of the interneuron(s) shifting its membrane potential away from the firing threshold. When cell 151 was hyperpolarized the AE response to P cell stimulation was similar to that observed in the presence of high concentrations of divalent cations. In addition, the 151 cells received, also, excitatory input from P cells (Fig. 5).

Thus, the sensory signal was channeled to the AE motorneuron through two main parallel pathways, a monosynaptic and a polysynaptic synapse. Due to its connectivity, cell 151 could be in the pathway between P and AE neurons, and it could modulate the relative expression of the hyperpolarizing and the depolarizing responses to the sensory input. It remains to be studied what are the inputs that regulate the activity of cell 151.

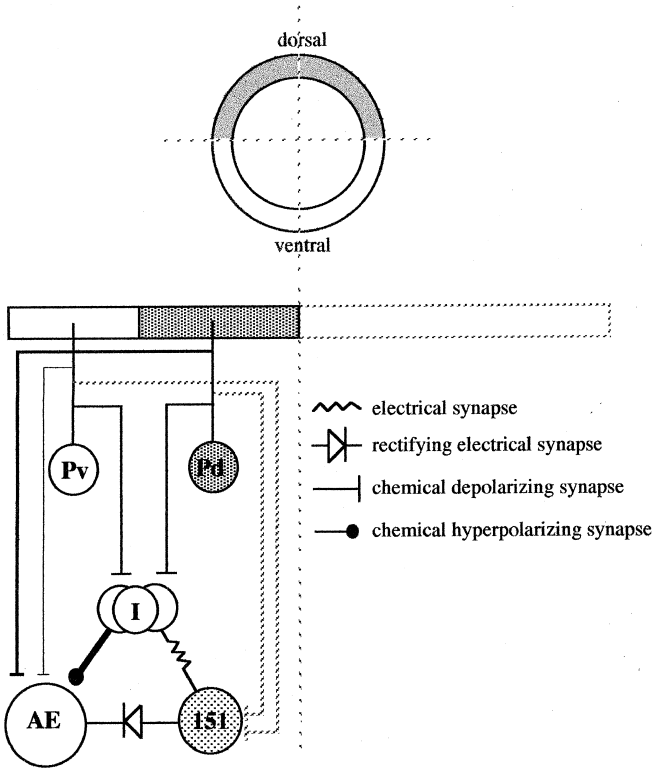

Fig. 6 Synaptic connectivity between the P and the AE neurons. The figure summarizes the interactions between the neurons P, AE and 151 within a hemiganglion, as interpreted from the results presented in this report. The scheme on the top represents a transversal section of the leech body, the dorsal skin is colored in gray. The neurons are represented as *circles* and the thickness of the lines indicates relative strength of the synaptic interactions. The unknown interneuron(s) is denoted as *I*. *Dotted lines* between the P and 151 cells indicate that these synaptic interactions have not been described in the present study, but preliminary results indicate that these interactions are mediated by chemical synapses

Physiological implications of the P-AE interactions

The two phases of the AE responses showed differential sensitivity to P_v and P_d cells. This pattern of sensory mapping by the AE neurons suggests that the two phases of the response may subserve different behaviors. The depolarizing response was evoked, primarily, by the P cells that innervate the dorsal aspect of the leech body wall. Because this phase of the response remained subthreshold, its effect must be confined to areas of the dendritic arbor close to the signal input site. No obvious physiological function can be ascribed to the depolarizing response at the moment. The hyperpolarizing response was more effectively evoked by P cells located contralateral to the AE cell body, irrespective of the dorsal versus ventral location of the stimulus. Since the AE motorneurons innervate the muscles erecting the annuli in the contralateral hemisegment, stimulation of one side of the body wall would produce a stronger annuli relaxation on that same side.

The mechanosensory P neurons respond to pressure exerted on the skin of the animal and their firing frequency depends on the pressure magnitude (Nicholls and Baylor 1968). These neurons represent a sufficient sensory drive to motor behaviors such as local shortening (Wittenberg and Kristan 1992), local bending (Kristan 1982), and swimming (Debski and Friesen 1987), and the firing frequency necessary to drive these behaviors was between 10 and 20 Hz. This same stimulus produced an effective inhibitory response on the AE motorneurons. Preliminary results in body-wall preparations ($n = 2$) showed that departing from a condition of erected annuli – depolarized AE neurons – a train of ten action potentials at 15 Hz in the P cell produced a visible relaxation of the annuli. The state of the annuli could affect the dynamic properties of the body wall and thus modulate the execution of motor reflexes, as in fact occurs during swimming (Friesen 1981); it could also affect the receptiveness of other sensory stimuli such as water vibration or light through the sensilla. These hypotheses are under current investigation.

Taken together the results show that even at the level of a single pair of sensory and motor neurons the circuitry is composed of multiple parallel pathways, each of which can affect the final motor performance, depending on internal and external influences, allowing for a more subtle regulation of the motor output.

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