Inhibitory neuron produces heterosynaptic inhibition of the sensory-to-motor neuron synapse in *Aplysia*

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We have identified an inhibitory neuron (RPL4) in the right pleural ganglion of *Aplysia*, which produced hyperpolarization of the sensory and motor neurons involved in the tail withdrawal reflex. Activation of RPL4 significantly reduced the amplitude of excitatory postsynaptic potentials produced in tail motor neurons by action potentials triggered in sensory neurons. This example of heterosynaptic inhibition was due, at least in part, to an increase in membrane input conductance in the motor neuron. Since the synaptic strength of the sensory-to-motor neuron connection has been associated with the strength of the tail withdrawal reflex, RPL4 may contribute to modulation of that reflex.

The mollusc *Aplysia* responds to tactile or electrical stimulation of the body wall with reflexive withdrawal of the siphon and tail. These defensive reflexes and the neural circuitry underlying them are modulated by both facilitatory and inhibitory processes. Extensive work has shown that facilitatory processes can increase the strength of the sensory-to-motor neuron synapse, thus potentiating defensive reflexes. It is also clear that various inhibitory processes may play a role in modulation of the neural circuitry underlying these reflexes. For example: (1) tail sensory neurons receive hyperpolarizing input which can be triggered by stimulation of the tail outside the receptive field of the sensory neuron, (2) the neuropeptide FMRFamide produces hyperpolarization and spike narrowing in sensory neurons, (3) bath application of FMRFamide as well as activation of an FMRFamidergic neuron produce presynaptic inhibition of the siphon sensory-to-motor neuron synapse. It has been suggested that this form of presynaptic inhibition contributes to behavioral inhibition of the siphon withdrawal reflex. In this report we describe an inhibitory neuron that could contribute to the hyperpolarization observed in the tail sensory neurons and play a role in inhibiting the tail withdrawal reflex. We will refer to this cell, which is located in the right pleural ganglion, as RPL4, continuing the established system for this ganglion.

*Aplysia californica* weighing 150–300 g were anesthetized with a volume of isotonic MgCl₂ equivalent to half the animals' weight. The animals were dissected and the right pleural-pedal ganglia were removed and pinned to a Sylgard coated chamber containing artificial sea water. Sensory and motor neurons of the isolated right pleural-pedal ganglia were identified and recordings were made using previously described methods. RPL4 was identified by (1) its location anterior to the sensory neuron cluster between the pleural-abdominal and pleural-cerebral connective (Fig. 1), (2) its relatively large diameter (100–200 μm), (3) a resting membrane potential between -40 and -55 mV, (4) little or no spontaneous synaptic input or spike activity, (5) its ability to produce hyperpolarization of a tail sensory and/or motor neuron, (6) the presence of both an antidromic action potential and excitatory synaptic input in response to electrical stimulation of nerve P9.

A burst of spikes in RPL4 leads to hyperpolarization of both the sensory and motor neurons (Fig. 2). The hyperpolarization of the motor neuron was generally more pronounced than that of the sensory neuron. A single spike in RPL4 produced a unitary inhibitory postsynaptic potential (IPSP) of constant latency in the motor neuron. The RPL4-induced hyperpolarization of the tail sensory and/or motor neuron, (6) the presence of both an antidromic action potential and excitatory synaptic input in response to electrical stimulation of nerve P9.

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We next examined whether RPL4 modulated synaptic transmission between the sensory and motor neurons (Fig. 3). Six action potentials were elicited in a sensory neuron at 60-s intervals. Approximately 400 ms before the fourth action potential, RPL4 was stimulated with a suprathreshold 900-ms depolarizing pulse (Fig. 3A2). Activation of RPL4 produced a hyperpolarization of the motor neuron and a decrease in the amplitude of the excitatory postsynaptic potential (EPSP). The EPSPs recovered after stimulation of RPL4 was stopped (Fig. 3A3). In this experiment there was not a significant hyperpolarization of the sensory neuron. Figure 3B illustrates average amplitudes of the EPSPs from the experimental group (n = 11) and from the control group (n = 13) in which RPL4 was not activated. The control group consisted of pooled data from sensory neurons (SNs) in different preparations (n = 10) and from different SNs in the same preparation as the SNs from the experimental group (n = 3). The amplitudes of the EPSPs produced during activation of RPL4 were significantly reduced compared to the control (t_{22} = 2.77; P <
Fig. 3. A: six action potentials were elicited in a sensory neuron with a 60-s interval (the third, fourth and fifth action potentials are shown). Prior to the fourth action potential, RPL4 was activated (A2) by a 900-ms suprathreshold depolarizing current pulse. Activation of RPL4 led to hyperpolarization of the motor neuron and decreased the amplitude of the EPSP produced by the action potential in the sensory neuron. B: average amplitudes of the EPSPs from the experimental group in which RPL4 was activated prior to the fourth stimulus, and from a separate control group of experiments, in which RPL4 was not activated at all. The asterisk indicates a significant difference between the experimental and control groups at the fourth stimulus (P < 0.02). The amplitudes of the EPSPs were normalized to the value of the first EPSP (average value of first EPSP was 3.7 ± 0.6 mV for the control group and 5.2 ± 1.0 mV for the experimental group; the difference was not significant, t2z = 1.32).

0.02). The inhibitory effect on the EPSPs is short-lasting, however, as there was no difference in the amplitude of the EPSPs elicited by the succeeding action potential 60 s later. In this series of experiments, the analysis was restricted to cases in which RPL4 produced a hyperpolarization of at least 2 mV in the motor neuron. (Approximately 35% of all RPL4-to-motor neuron (MN) connections met this criterion.)

An example of the degree to which this form of inhibition can affect the processing of information in this circuit is shown in Fig. 4. In this experiment the sensory-to-MN connection was sufficiently strong to elicit an action potential in the MN (Fig. 4A). Activation of RPL4, however, was sufficient to block the action potential in the MN (Fig. 4B). The absence of the action potential in the MN was not due to synaptic depression caused by repetitive activation of the SN–MN synapse, since the following spike in the SN was still able to trigger an action potential in the MN (Fig. 4C).

The inhibition produced in the MN by RPL4 is due to both hyperpolarization and an increase in membrane input conductance. The latter is probably primarily responsible for modulating the amplitude of the EPSPs by shunting the postsynaptic current. Hyperpolarization by itself increases the amplitude of the EPSP (unpublished observations). We cannot, however, eliminate the possibility that RPL4 produces some form of presynaptic inhibition of the SN–MN connection. We believe it is unlikely that there is a large presynaptic contribution, since the amplitudes of the EPSPs were not consistently reduced in those preparations in which the SN was hyperpolarized by activation of RPL4 but the MN was not (data not shown).

Activation of LPL16, an FMRFamidergic neuron located in the left pleural ganglion, inhibits synaptic transmission of the siphon sensory-to-MN synapses located in the abdominal ganglia. We believe that RPL4 is not a contralateral homologue of LPL16 because the two cells appear to have functionally distinct effects on their target cells. Both LPL16 and bath application of FMRFamide seem to decrease EPSP amplitude in the abdominal SNs via a presynaptic mechanism, whereas the synaptic inhibition of the pleural-pedal sensory-to-MN synapse produced by RPL4 seems to be largely postsynaptic. Moreover, preliminary immunocytochemical experiments indicate that RPL4 is not FMRFamidergic. It is likely that RPL4 is one of the non-FMRFamidergic cells identified by Xu et al.21.

The function of RPL4 in the tail-withdrawal reflex is not well understood. It could contribute to the hyperpo-
larization of SNs observed by stimulation of the tail outside the receptive field of the SN. Indeed, in a single experiment using a semi-intact preparation we found that stimulation of the tail with a von Frey hair produced bursts of activity in RPL4, indicating that it receives sensory input and could account for the hyperpolarization observed in SNs that are not activated by the stimuli. Short-lasting inhibition of the siphon withdrawal reflex has been demonstrated in response to noxious stimulation. An inhibitory interneuron (L16) in the abdominal ganglion, which inhibits siphon SNs and MNs, appears to be essential for inhibition of the siphon withdrawal reflex. Similarly, it is possible that activation of RPL4 and thus inhibition of the SNs and MNs could account for a similar phenomenon in the tail-withdrawal reflex. Indeed, we noted that RPL4 would often fire for a few minutes in response to a train of stimuli to nerve P9. Inhibition of the tail could also be mediated by inhibition of excitatory interneurons in the reflex pathway (e.g. Wright et al.20).

Our results represent a first step towards understanding the role of inhibitory neurons in the modulation of the tail-withdrawal reflex. These may be important not only for understanding the reflex but also for analyzing the components of several forms of learning, including habituation, dishabituation, sensitization and classical conditioning.

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