Coactivation of Putative Octopamine- and Serotonin-Containing Interneurons in the Medicinal Leech

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Gilchrist, Laura S. and Karen A. Mesce. Coactivation of putative octopamine- and serotonin-containing interneurons in the medicinal leech. J. Neurophysiol. 78: 2108–2115, 1997. Possible interactions between octopamine-immunoreactive (IR) and serotonergic neurons in the CNS of the medicinal leech were investigated. Simultaneous intracellular recordings of serotonin-containing neurons (either the Retzius neuron or cell 21) and the dorsolateral octopamine-IR (DLO) neuron demonstrated that both sets of neurons are coactive at times. Depolarization of either serotonergic cell 21 or the Retzius neuron did not alter the membrane potential of the DLO. Similarly, depolarization of the DLO did not affect the serotonergic neurons examined. Because it was found that the DLO and either the serotonergic cell 21 or Retzius neuron were at times coactive, we looked for possible sources of common excitatory inputs. The centrally located pressure (P)- and touch (T)-sensitive mechanosensory neurons excited the DLOs through a polysynaptic pathway. Stimulation of nociceptive (N) mechanosensory neurons did not cause a measurable depolarization in the membrane potential of the DLO. Through simultaneous recordings of the DLO, cell 21, and a particular identified mechanosensory neuron, it was demonstrated that activity in the T or P cells can excite both serotonergic cell 21 and the octopamine-IR DLO. These findings indicate that, in many instances, both serotonin and octopamine, biogenic amines with neuromodulatory actions in many different invertebrates, may be released simultaneously in the leech.

INTRODUCTION

Biogenic amines have been shown to act as neuromodulators, neurohormones, and neurotransmitters in a number of invertebrate preparations (reviewed in David and Coulon 1985; Evans 1980, 1985). Much attention in invertebrate neurobiology has centered on two biogenic amines, octopamine and serotonin. Octopamine is the phenol analogue to norepinephrine found in both invertebrates and vertebrates, but its function has been investigated more widely in the invertebrates. Dramatic behavioral changes can occur when the levels of serotonin or octopamine are altered either naturally or artificially. A few examples of how changes in the biogenic amines correlate with behaviors include: aggressive activity in crickets (Adamo et al. 1995), behavioral class types in honeybees (Taylor et al. 1992), and feeding in leeches (Lent and Dickinson 1984; Sabban and Kleinhaus 1994). Artificial alterations in the levels of octopamine or serotonin also can result in significant behavioral changes (for examples, see Lent et al. 1991; Livingstone et al. 1980; Monastirioti et al. 1996). One of the most dramatic alterations is observed when serotonin or octopamine is injected directly into the hemolymph of the lobster. In these experiments, serotonin produces aggressive behavioral posturing, whereas octopamine injection results in the expression of a submissive postural stance (Livingstone et al. 1980). The clear functional antagonism of serotonin and octopamine in this set of experiments has driven a number of scientists to look for such antagonism in other systems.

It remains unclear, however, if this striking behavioral antagonism prevails in other animals using octopamine and serotonin. In the cricket, octopamine levels seem to rise in animals engaged in an aggressive behavioral encounter whether or not the individual wins the encounter or becomes the subordinate (Adamo et al. 1995). In the leech nervous system, there appears to be some similarity in function of the two neurotransmitters. Hashemzadeh-Gargari and Friesen (1989) found that either serotonin or octopamine can enhance the expression of swimming motor patterns, although serotonin was more effective. In two studies (Belardetti et al. 1984; Catarsi et al. 1995), either serotonin or octopamine application was shown to reduce the afterhyperpolarization of the touch mechanosensory cells, although the underlying mechanisms may differ.

To determine if there were any interactions, either antagonistic or synergistic, between octopaminergic and serotonergic neurons in the leech CNS, we decided to undertake a circuit analysis of the well-studied serotonergic neurons (Lent et al. 1991; Nusbaum and Kristan 1986; Rude 1969), and a pair of recently identified dorsolateral octopamine-immunoreactive (IR) neurons (DLOs) (Gilchrist et al. 1995). If one assumes that serotonin and octopamine act antagonistically at the organismal and cellular level in the leech, then this should be reflected in the aminergic cells not firing together or even being reciprocally inhibitory.

Thus we recorded simultaneously from the DLO and one of two identified serotonergic neurons, the Retzius neuron and cell 21. Our findings indicate that these two sets of neurons do not inhibit each other and instead sometimes are active simultaneously. Having established that the octopamine and serotonin interneurons are at times simultaneously active, we then investigated the nature and source of the coordinated activation of the DLO and cell 21. We determined whether the activation was due to excitatory connections between the octopamine- and serotonin-containing neurons or was due to simultaneous activation originating from other neurons. In particular, we focused on the synaptic inputs from identified mechanosensory neurons, cells previously known to activate serotonergic interneurons (Nusbaum and Kristan 1986).
METHODS

Animals

Experiments were performed on adult Hirudo medicinalis obtained from Leeches USA (Westbury, NY). Leeches were maintained in artificial pond water (0.5 g/l Hirudo-salt from Biopharm, Charleston, SC) at room temperature.

Electrophysiological recordings and cell identification

Adult leeches were anesthetized by cooling on ice for 10–15 min and then dissected in cold normal leech saline [which contained (in mM) 115.0 NaCl, 1.8 CaCl₂, 4.0 KCl, and 10.0 Tris-maleate] (Nicholls and Baylor 1968). All experiments were performed on preparations taken from the posterior ganglia of the nerve cord, G 14–G 21.

For both recording and dye iontophoresis, electrodes tips were filled with 5% Neurobiotin (Vector Laboratories, Burlingame, CA) dissolved in 2 M potassium acetate. Glass microelectrodes were pulled to resistances between 40 and 60 MΩ when filled with Neurobiotin at the tips and back filled with 2 M potassium acetate. Iontophoretic injection was accomplished with a constant depolarizing current of 1–2 nA (Kita and Armstrong 1991). All cells that were recorded from were iontophoretically injected with Neurobiotin then later reacted with an antibiotic antibody to confirm cell identity (Gilchrist et al. 1995). Intracellular signals were amplified through a Cornerstone IX2-700 (Dagan, Minneapolis, MN) electrometer and displayed on a Tektronix 5113A storage oscilloscope (Tektronix, Beaverton, OR). In addition, some intracellular signals were recorded using a MacLab Chart program running on a PowerMacintosh computer with a sample rate of 200/s.

Identification of the DLOs and cells 21 was facilitated by submerging the intact leech in a 0.005% solution of Neutral red (Sigma, St. Louis, MO) for ≥2 h (Lent 1981). Both the DLO and cell 21 stained red, but only in midbody ganglia 7–14 does the DLO not become stained (Gilchrist et al. 1995). Identification of the DLO for all experiments was determined both by morphology and colocalization of octopamine-immunoreactivity with the intracellular Neurobiotin. The main branching pattern of the DLO consisted of a primary neurite that extended across the midline and turned into a single ascending process, which projected through the contralateral connective (Gilchrist et al. 1995). Serotonergic cells were identified by their unique cell positions and morphologies (Lent 1981; Nusbaum and Kristan 1986).

Coactivation of serotonin and octopamine neurons

In these experiments, one DLO was impaled with a glass microelectrode, whereas a second electrode was placed in a serotonergic neuron. In all experiments, two contiguous posterior ganglia were used. Recordings were made from DLOs and cells 21 in the same and adjoining ganglia, whereas recordings of the Retzius cells and DLOs could be obtained only in separate but adjoining ganglia due to their placement on opposite (ventral vs. dorsal) sides of the ganglion.

Sensory input to the DLOs

Two adjacent posterior ganglia were dissected out of the leech. Given that most of the mechanosensory neurons are located ventrally and the octopamine-IR DLOs are located dorsally, recordings were conducted in separate but connected ganglia (see Fig. 1). Except where noted, all recordings were done with preparations in normal leech saline. In all figures, the capacitance artifacts (marked by arrows) indicate the on- and off-set of the depolarizing pulse.

To test for monosynapticity, the normal saline was replaced with 20 mM Mg²⁺/20 mM Ca²⁺ saline (Nicholls and Purves 1970). Additionally, spike triggering was used to look for jitter in the timing of activation of the DLO.

FIG. 1. Schematic drawings of the experimental setup for dual intracellular recordings. Dorsal neurons in the right hemisphere appear on the viewer’s left. All cells diagrammed are bilaterally paired even when shown as singlets. A: experimental setup used for both the Retzius cell—dorsolateral octopamine-immunoreactive neuron (DLO) and the mechanosensory cell—DLO recordings. In these 2 sets of experiments, 1 ganglion was pinned ventral side up, allowing for access to Retzius cells and mechanosensory cells; the other ganglion was pinned dorsal side up allowing for impalement of the DLO. B: configuration of ganglia used during pair-wise recording of the DLO and serotonergic cell 21. Cells were impaled either in the same or adjacent ganglia.

Immunocytochemistry and microscopy

Tissue was fixed according to the methods of Gilchrist et al. (1995). The DLOs were labeled with a polyclonal antibody raised against an octopamine-glutaraldehyde-thyroglobulin complex in rabbit (Eckert et al. 1992; Gilchrist et al. 1995). Ganglia were incubated in a 1:200 dilution of the octopamine antibody in 1% Triton X-100 buffer for 4 days at 4°C. After a 1 day wash at 4°C, the tissue was incubated for 48 h in a goat anti-rabbit antibody conjugated with the cyanine 5.18 (Cy5) fluorophore (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:50 in 1% Triton X-100 buffer (Mesce et al. 1993). Additional protocols were also reacted with a solution of 1:100 streptavidin conjugated with the cyanine 3.18 (Cy3) fluorophore (Jackson Immunoresearch) to label the intracellular Neurobiotin (Mesce et al. 1993). Tissue was washed, dehydrated, and cleared following the protocols of Gilchrist et al. (1995).

Immunostained samples were viewed using a Bio-Rad MRC-600 or MRC-1000 laser scanning confocal microscope (Bio-Rad Life Science Division, Hercules, CA). The excitation source was a 15-MW mixed-gas krypton/argon laser (ILT model 5470K, Ion Laser Technology, Salt Lake City, UT). The use of the multiline method of visualization, which allowed for the complete separation of Cy3 from Cy5, is reviewed in Brelje et al. (1993) and Mesce et al. (1993). Ganglia were imaged using Nikon Fluor ×10, ×20, and ×40 objectives. A Kodak XL 700 Digital Color Printer (Rochester, NY) with a resolution of 2,048 × 1,536 pixels was used to produce prints in this paper.
RESULTS

Simultaneous recordings of the DLO and either cell 21 or the Retzius cell

Do the serotonergic and octopamine-IR interneurons in the leech communicate with each other? To investigate this question, we recorded intracellularly from identified interneurons in the CNS of adult H. medicinalis. Specifically, we recorded from the octopamine-immunoreactive DLO and identified serotonergic neurons to look for possible connections or interactions.

In the first set of experiments, we recorded from the serotonergic Retzius neuron and the DLO in adjoining ganglia. The experimental set-up for this and several other dual intracellular recordings is diagrammed in Fig. 1. As can be seen in Fig. 2A, the DLO and Retzius cells were, at times, coactive (n = 12). In each of these preparations, the two cells repeatedly would go from being relatively silent to firing short bursts of spikes together, or both cells would simultaneously increase their rate of tonic firing. At other times, however, one cell would fire spikes while the other remained silent. Stimulation of the DLO, however, did not result in activation of the Retzius cell and reciprocal stimulation of the Retzius cell did not depolarize the DLO (Fig. 2B). Because the Retzius cell is located on the ventral surface and the DLO resides on the dorsal surface of the ganglion, we were not able to record from DLOs and Retzius cells within a single ganglion. Recordings were obtained sometimes from a Retzius cell in the anterior ganglion and a DLO in the posterior ganglion of the preparation, whereas at other times, the positions of the recorded cells were switched. Additionally, we recorded from cells residing on the same and opposite hemispheres of the two ganglia. No difference in activation was seen when positions of the two cells were varied.

Next, we recorded from the DLO and cell 21. Two adjoining ganglia were used as in the previous configuration, but, in this case, we were able to record from the serotonin and octopamine cells within a single ganglion because both have dorsal somata (Fig. 1B). As was the case with the Retzius cell and DLO recordings, the DLO and serotonergic cell 21 increased their activity together (Fig. 3A). Importantly, electrical activation of one cell did not result in excitation of the other (Fig. 3B; n = 13). In these experiments, we were able to ascertain if each of the two cells within a single ganglion influenced the activity of the other. Additional experiments were conducted in which we recorded from cells in adjoining ganglia similar to the configuration used for the dual recordings of DLO and Retzius neurons. We sometimes recorded from two contralaterally located somata, whereas at other times, ipsilaterally located somata. In both cases, no difference in activation was seen when the position of the two recorded cells was varied (data not shown). Thus although particular serotonergic neurons are active at approximately the same time as the DLOs, we observed no excitatory connections between the two sets of neurons that can account for their coactivation.

Mechanosensory inputs to the octopamine-IR neurons

Which cells excite the DLO, and what might be a source of common excitatory input to the octopamine-IR and serotonergic neurons? To identify inputs to the DLO during a series of pilot studies, we first used a preparation in which a part of the leech’s body wall and nerve roots were connected to the segmental ganglia (Nicholls and Baylor 1968). By touching the skin, we depolarized the DLO (personal observations).

Identified mechanosensory neurons, specifically the touch (T), pressure (P), and nociceptive (N) neurons, have been shown to innervate the skin (Nicholls and Baylor 1968). Thus we explored the possibility that these cells might activate the DLOs. Because these cells also are known to excite the serotonergic cell 21 (Nusbaum and Kristan 1986), they were a potential source for coactivation of the aminergic cells.

These large mechanosensory neurons, with somata located within the CNS, were identified both by their electrophysiological signature and cell position (Nicholls and Baylor...
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FIG. 3. Dual intracellular recordings from serotonergic and octopamine-IR neurons. A: intraganglionic (ganglion 15) intracellular recordings obtained from the DLO and cell 21 (right and left sides, respectively). Simultaneous onset of increased activity expressed by both the DLO and cell 21 (†). Although there was not a 1-to-1 correlation of spikes between the 2 cells, the onsets in activity were repeatedly similar. B: depolarization of cell 21 (†), through positive current injection, did not alter the electrical activity of the DLO. C: reciprocal experiment to that shown in B, in which electrical activation of the DLO did not alter the activity of cell 21.

1968). The experimental preparation consisted of two adjoining ganglia (Fig. 1A) in which we stimulated individual mechanosensory cells using intracellular electrodes.

T cell

Stimulation of touch mechanosensory (T) cells, located in ganglia anterior to and posterior to a given DLO, caused excitation of the DLO in >90% of all preparations (n = 19). We used two criteria to determine if the connection between the T cells and the DLO was monosynaptic or polysynaptic. First, using spike triggering, we looked for “jitter” in the delay of the DLO’s activation to the sensory cell spikes. Second, we used high Mg²⁺/Ca²⁺ saline to look for loss of activation. If the synapse were polysynaptic, there would be jitter in the delay, and the connection between the sensory cell and the DLO would be lost in high Mg²⁺/Ca²⁺ saline.

All recordings showed jitter in the delay between the T cell action potential and DLO response. In all preparations tested (n = 3), excitatory input from the T cell was blocked in high Mg²⁺/Ca²⁺ saline. In all cases, excitatory input resumed once the high Mg²⁺/Ca²⁺ saline was washed thoroughly out (data not shown). These results indicate that the connection between the T cell and the DLO is polysynaptic.

To demonstrate directly that the T mechanosensory neuron could activate both these serotonergic and octopamine-IR neurons, we impaled a T cell in one ganglion, and cell 21 and the DLO in the other, as illustrated in Fig. 4A. In all preparations (n = 3), both the DLO and cell 21 depolarized in response to depolarization of the T cell (Fig. 5). Subsequent morphological confirmation of triple cell identity can be seen in Fig. 4, B and C, which shows confocal images of Neurobiotin fills of the T mechanosensory cell, the DLO, and cell 21.

P cell

Activation of both medial and lateral P cells in ganglia anterior and posterior to the DLO (n = 25). In 56% of all preparations tested, stimulation of the P cell caused a depolarization of the DLO (Fig. 6). In the remaining 44% of preparations, activation of the P cell resulted in no change in DLO membrane potential. P cell identity (medial vs. lateral) was confirmed through cell morphology, and P cell position was not correlated with DLO activation. Instead, the activity level of the P cell was a factor in determining if depolarization of the P cell resulted in a visible change in the DLO’s membrane potential. When
the P cell responded to the depolarizing stimulus pulse with three or more action potentials, 14 of the 17 DLOs depolarized. Of the preparations where the P cell responded with only one or two action potentials, seven out of eight DLOs did not depolarize.

The P cell to DLO connection also was tested for monosynapticity using high Mg\(^{2+}/Ca^{2+}\) saline. In all preparations (n = 3), the excitatory connection was blocked in high Mg\(^{2+}/Ca^{2+}\) saline. The excitatory connection was restored in all preparations on washout (data not shown), indicating a polysynaptic connection.

To test directly whether the P mechanosensory neuron could cause coactivation of the DLO and cell 21, triple-cell recordings were conducted as described for T cell activation. Stimulation of the P cell resulted in activation of both the DLO and cell 21 in two out of the three preparations tested (Fig. 6). In the remaining preparation, only cell 21 responded to P cell input, whereas the DLO did not. This is consistent, however, with our previous results in which P cell activation of the DLO was variable.

**N cell**

Stimulation of nociceptive (N) cells in ganglia anterior or posterior to a given DLO did not cause any change in the activity level of the DLO (Fig. 7; n = 15). To assure ourselves that our preparations were viable enough to sustain normal synaptic connections, input from the T cell to the DLO also was tested. In five preparations where N cell activation did not change the level of DLO activity, intracellular activation of the T cell did depolarize the DLO. Additionally, in one preparation, an inhibitory post synaptic potential was observed in the DLO in response to most all of the action potentials of the N cells (data not shown).

**DISCUSSION**

Coactivation of serotonin and octopamine cells

Octopamine and serotonin are biogenic amines with reported neuromodulatory effects in the leech and a number of other invertebrates (Evans 1978; Hashemzadeh-Gargari and Friesen 1989; Lent and Dickinson 1984; Willard 1981). In the present study, we examined the possible synaptic connections between two sets of interneurons expressing these biogenic amines in the leech CNS. Although serotoninergic neurons long have been identified in the leech CNS and much is known about their synaptic connections, relatively little is known about putative octopaminergic neurons. The recently identified dorsolateral octopamine-IR interneurons, the DLOs, were observed in segmental ganglia 1–6 and 15–21, but not in midbody ganglia 7–14 (Gilchrist et al. 1995). We confined our study to a subset of DLOs, present in posterior ganglia 15–21. Thus the possibility remains that the DLOs in anterior ganglia express unique relationships with the anterior serotoninergic cells and mechanosensory neurons that are distinct from the observations reported here.

The physiological antagonism of octopamine and serotonin, so powerfully demonstrated in the lobster (Livingstone et al. 1980), has not been observed in the leech, yet the relationship between these two neurotransmitter systems is
still intriguing. In contrast to what might be expected from the work in lobsters, the two sets of neurons did not exhibit reciprocal inhibitory connections (Figs. 2B and 3B). These results are consistent with the observations of others that, in the leech, serotonin and octopamine may not have antagonistic roles (Belardetti et al. 1984; Catarsi et al. 1995). A major observation of the present study was that both sets of serotonergic cells examined, the Retzius neuron and cell 21, can be coactive with the octopamine-IR DLOs (Figs. 2A and 3A). The significance of the coactivation of these sets of neurons, however, is not clear as we have not yet identified the postsynaptic connections of the DLOs. Yet, it does demonstrate that the release of these two neurotransmitters is at times temporally linked via the activation of common excitatory inputs. Revealing such relationships adds to our understanding of whether patterns of serotonin and octopamine release might be correlated with antagonistic or cooperative effects.

It should be emphasized that the DLOs and the two sets of serotonergic neurons examined here, the Retzius neuron and cell 21, are not always coactive. In a number of preparations, there were times when the two sets of cells did not fire together. As we later demonstrated with the mechanosensory cell experiments, although these neurons share a collection of common excitatory inputs, there are differences in postsynaptic inputs.

In addition to the Retzius cell and cell 21, there are three additional sets of serotonergic neurons in the CNS of the leech: the AM and PM cells found only in anterior ganglia and the paired cells 61 found throughout the nerve cord (Stuart et al. 1974). Because these cells were not included in our experiments, we cannot claim that all serotonergic neurons and octopamine cells should be coactive. It seems likely, however, that cell 61 and the DLO are coactive, as cell 61 receives excitatory input from the same mechanosensory neurons as does cell 21 (Nusbaum and Kristan 1986).
Sensory input to the DLOs and serotonergic neurons

Understanding how octopamine influences a specific neural circuit depends, in part, on identifying those sensory inputs necessary for activation of putative octopaminergic neurons. Even more intriguing may be the revelation of which types of neuromodulators may be released in synchrony. Studies of a number of invertebrate preparations have demonstrated that numerous neuromodulators can influence the generation of rhythmic motor patterns (Hashemzadeh-Gargari and Friesen 1989; Marder 1987) and have led researchers to ask what their different roles may be and how, if at all, they influence one another (Dickinson 1989; Dickinson et al. 1997).

In the experiments presented here, we aimed to identify and begin to understand better the sensory inputs to the putative octopaminergic interneurons, the DLOs. We demonstrated that identified mechanosensory neurons excite the DLOs via polysynaptic pathways. Stimulation of the T mechanosensory cells produced the most reliable and robust excitation of the DLO (Fig. 5). P mechanosensory cells also were found to stimulate the DLO via a polysynaptic pathway (Fig. 6). The N mechanosensory neurons were found not to excite the DLOs (Fig. 7), although there was a hint of inhibitory input (data not shown). The lack of excitation was not correlated to unhealthy preparations.

Bursts of action potentials in the P cells, which occur in response to natural stimuli (Nicholls and Baylor 1968), excited the DLO in nearly all cases. Only in those trials in which the depolarizing pulse resulted in the production of one or two P cell action potentials were no excitatory responses seen in the DLO. Thus the variability in the DLO’s response to P cell input appears to relate to the number of action potentials generated by the P cell. We found no correspondence between P cell position or morphology and its ability to excite the DLO.

According to our findings thus far, it appears that the primary mechanosensory activation of the DLO is through T and P cell stimulation and not N cell input. These results form an interesting contrast with what is known about the serotonergic interneuron, cell 21. According to Nusbaum and Kristan (1986), cells 21 and 61 respond best to P cell excitation and next best to N cell excitation, T cell activation resulted in the weakest excitatory response. Thus based on the patterns of P, T, and/or N cell activation, which in turn correspond to the characteristics of a natural stimulus, it is possible that slightly different concentrations of the two biogenic amines will be released in the CNS. Small variations in these concentrations may then alter the output of neural networks.

The experiments reported here suggest that, in many cases, a combination of both serotonin and octopamine will be released in the CNS. Most studies exploring the effects of neuromodulators on neural circuits, understandably for simplicity sake, have used single neuromodulators. Some of these previous experiments have even shown that serotonin and octopamine have similar effects (Catarsi et al. 1995; Hashemzadeh-Gargari and Friesen 1989). It now might be of interest to look at the effects of combinations of these two neurotransmitters/neuromodulators in preparations where they have been previously tested alone.

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