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Avermectin B_{1a}, a paralyzing anthelmintic that affects interneurons and inhibitory motoneurons in *Ascaris*

(parasites/nematodes/synaptic activity/pharmacology/avermectin)

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ABSTRACT **Avermectin B_{1a} (AVM) is an antiparasitic agent that paralyzes nematodes without causing hypercontraction or flaccid paralysis. Using selective stimulation techniques, we have shown that AVM blocks transmission between interneuron(s) and excitatory motoneurons in the ventral nerve cord of *Ascaris*. It also inhibits transmission between inhibitory motoneurons and muscle but has little effect on excitatory neuromuscular transmission. Picrotoxin can reverse the AVM-induced block of interneuron–excitatory motoneuron transmission but has no effect on the inhibitory motoneuronal synapse in either the presence or absence of AVM. Our results provide an explanation of how AVM may cause paralysis of nematodes.**

Avermectin B_{1a} (AVM) is a macrocyclic lactone derived from the mycelia of *Streptomyces avermilitis* (1–3). It is a potent broad-spectrum anthelmintic and insecticidal agent (4, 5) but has no demonstrated antibacterial or antifungal activity (2). When applied to the neuromuscular junctions of lobster walking legs, the drug rapidly eliminated the inhibitory postsynaptic potentials and then more slowly brought about a reduction in the amplitude of excitatory postsynaptic potentials (6). The input resistance of the muscle fibers also decreased, with a time course similar to that of the reduction of excitatory potentials (6). None of these effects was reversible by washing, but the reductions in the excitatory potentials and input resistance were reversed by picrotoxin. It was postulated that, at the lobster neuromuscular junction, AVM acts to open the chloride channels (6). This could be accomplished by AVM directly or by an AVM-stimulated release of γ-aminobutyric acid (GABA) from the inhibitory neuron. AVM has been shown to cause release of GABA from mammalian synaptosomes (7). In binding studies using mammalian brain tissue, no competition was seen between AVM and GABA, showing that, at least in mammals, AVM and GABA do not interact with identical binding sites (8).

In the present studies we investigated the mechanism of action of AVM on nematodes. Both the large parasitic nematode *Ascaris* and the small free-living nematode *Caenorhabditis* were used for behavioral studies. Electrophysiogical studies were carried out on *Ascaris*. The effects of AVM were tested in dissected preparations in which single excitatory or inhibitory motoneurons were activated, either directly by electrical stimulation or indirectly by stimulating interneurons that synapse onto the excitatory motoneurons. We find that AVM inhibits transmission between interneurons and excitatory motoneurons. It also inhibits transmission between inhibitory motoneurons and muscle but has little effect on excitatory neuromuscular transmission. The effect of AVM on the interneuron-to-excitatory motoneuron pathway was reversed by picrotoxin, but the action of the drug on inhibitory neuromuscular transmission was not.

MATERIALS AND METHODS

Nematodes. *Caenorhabditis elegans* strains N2 (wild-type) and E1072 (levamisole resistant) were obtained from J. A. Lewis (Columbia University). Stocks were maintained on petri plates at 18°C (9). *Ascaris suum* were collected from local slaughterhouses. They were transported to the laboratory in Kronecker's solution (0.9% NaCl/1.5 mM NaOH) at 37°C and kept at 37°C in this same solution or in *Ascaris* Ringer's solution (24.5 mM KCl/11.8 mM $CaCl_2$/9.8 mM $MgCl_2$/3.9 mM NaCl/125 mM NaOAc/5 mM Hepes, pH 7.4). *Dictyocaulus viviparus* and *Trichostrongylus colubriformis* were kindly provided from infected cattle by J. R. Egerton (Merck).

Chemicals. AVM, prepared by the methods of Miller *et al.* (1), was obtained from Merck Sharp & Dohme. It was stored frozen in dimethyl sulfoxide (Me_2SO) at −20°C. *Electrophorus electricus* acetylcholinesterase, acetylcholine chloride, GABA, neostigmine bromide, piperazine citrate, and picrotoxin were purchased from Sigma. Levamisole·HCl was obtained from the American Cyanamid Company. All the other chemicals were of the highest purity available from commercial sources.

Biochemical Assays. Lactic acid was determined by the lactate dehydrogenase/NAD assay by measuring NADH at 340 nm. Succinic, aspartic, and glutamic acids were isolated by thin-layer chromatography, and their radioactivities were measured in a liquid scintillation counter. Acetylcholinesterase activities were assayed by the procedure of Hestrin (10).

Length Measurements on *Ascaris*. Adult female *Ascaris* were injected with drugs dissolved in Ringer's solution. AVM was first dissolved in Me_2SO and then diluted with Ringer's solution. Drug solution (0.1 ml) was injected through the lateral line at a point 3–4 cm from the anterior end. As controls, injections of the same volume of Ringer's solution or of 10% Me_2SO (the highest concentration of Me_2SO injected with AVM) were performed. The animals were maintained at 37°C. Photographs of injected animals were enlarged to full size and measured with an electronic graphics calculator.

Dose–Response Data. Suspensions of *C. elegans* were incubated in Ringer's solution containing drugs for 10 min at room temperature, and the fraction of motile worms was counted under a dissecting microscope.

Anatomical Background, and Dissections for Electrophysiological Experiments. In the *Ascaris* motor nervous system there are seven different types of motoneurons: three dorsal excitors, one dorsal inhibitor, two (presumed) ventral excitors, and one ventral inhibitor (11). Tests were made on the effects of AVM on two of the seven types: DE1, one of the dorsal

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Abbreviations: AVM, avermectin B_{1a}; GABA, γ-aminobutyric acid; Me_2SO, dimethyl sulfoxide.

excitatory motoneurons; and VI, the ventral inhibitory motorneuron. The DE1 motoneuron has a ventral dendritic process that receives input from interneurons and a dorsal axon that makes synapses to muscle (Fig. 1*b*). The VI motoneuron has a dorsal dendrite receiving input from dorsal excitatory motoneurons and a ventral axon synapsing onto muscle (Fig. 1*c*). The branches of the motoneurons that connect their dorsal and ventral processes are called commissures (a commissure in *Ascaris* is a process of a single motoneuron) and are the only neural connections between the dorsal and ventral nerve cords in the body region of the animal.

In the body of *Ascaris* there are five repeating arrays of motoneurons, each containing 11 neurons. In each repeat, the DE1 and VI neurons occur twice. All of the electrophysiological experiments described in this paper utilized the anterior DE1 and VI motoneurons in the second repeating unit. The commissures of these cells were identified in live *Ascaris* by using a dissecting microscope and dark-field illumination. Their position was marked by carmine particles. The anteriormost 0.5 cm of the animal was then removed and a 3- to 5-cm length of worm containing the selected motoneurons was taken. By using the carmine marks as guides, this cylinder was slit open along the left lateral line; the right lateral line was then partially cut, leaving only one pair of commissures (consisting of one DE1 and one VI commissure) connecting dorsal and ventral halves (see Fig. 1*a*).

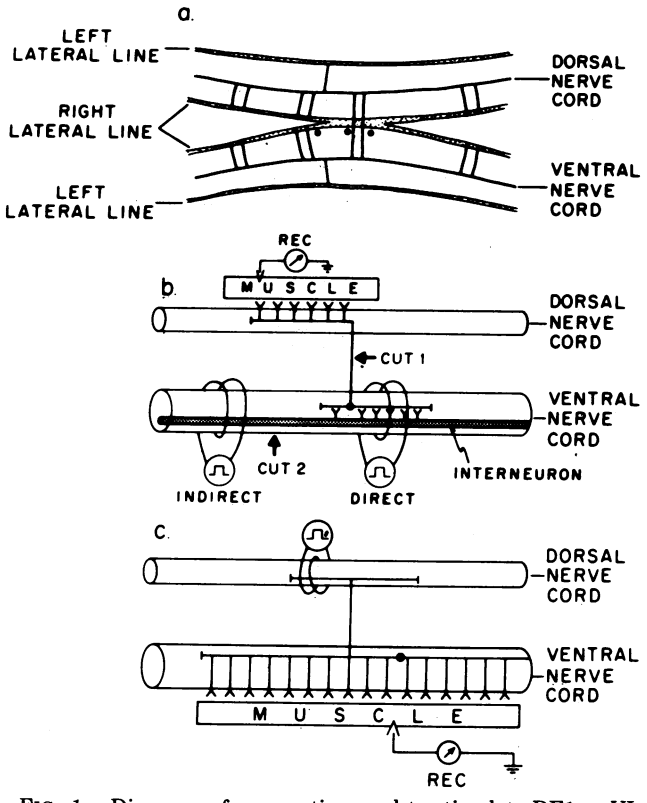

FIG. 1. Diagrams of preparation used to stimulate DE1 or VI motoneurons. (*a*) Portion of the body wall of *Ascaris* viewed from the inside (lateral lines stippled; muscle omitted). Nerve cords are represented by horizontal lines and the commissures, by vertical lines. The left lateral line is completely cut. The right lateral line has a bridge of tissue containing a DE1/VI commissure pair. The dots represent the position of carmine particles used to locate the commissures. (*b*) Cell body and processes of the DE1 motoneuron; interneurons are stippled. DE1 is activated by stimulating the ventral nerve cord either over its process (DIRECT) or anterior to its process (INDIRECT). (*c*) The VI motoneuron is activated by stimulating its dorsal process.

Electrophysiological Recordings. *Ascaris* Ringer's solution was heated with a jacketed water bath and perfused through the experimental chamber (volume, 5 ml) at approximately 12 ml/min. The temperature of the Ringer's solution in the chamber was maintained at 37°C. Drugs were added to the Ringer's solution in the jacketed water bath. There was a 2-min delay before the drug reached the chamber.

All recordings were made intracellularly from muscle cells by using glass micropipettes filled with 3 M potassium acetate and having a resistance of 20 to 60 MΩ. When AVM was applied to the bath, 1% Me_2SO was included in the solution to keep the drug soluble. The presence of 1% Me_2SO had no discernible effect on the electrophysiological recordings.

AVM is extremely hydrophobic and sticky; great care had to be taken to wash out the tubing and chamber after an experiment. The tubing was washed by passing 120 ml of 50% ethanol and then 120 ml of distilled water through the system with a syringe. The chamber was washed with similar solvents.

The DE1 motoneuron was stimulated through two bipolar electrodes located over the ventral nerve cord. The first electrode was positioned over the ventral branch of the DE1 motoneuron close to its cell body and the origin of its commissure; this electrode was used for direct stimulation. The second electrode was located over the ventral cord approximately 1.3 cm anterior to the DE1 motoneuron; stimulation here activated the DE1 motoneuron indirectly, presumably through an interneuron. The VI motoneuron was stimulated directly by a bipolar electrode placed over its process in the dorsal cord (Fig. 1*c*).

The DE1 neuron was stimulated at a rate of one per 4 sec, five times indirectly then five times directly. The preparation was allowed to rest for 10 min before this procedure was repeated. The stimulus variables (duration and intensity) for the direct and indirect stimuli were adjusted independently to give a maximal response. In many cases it was necessary to give a burst of stimuli (10–30 msec long; frequency adjusted for maximal response) to produce a response to indirect stimulation. In such cases these bursts were given every 4 sec. The VI neuron was stimulated continuously at a rate of one per 4 sec; the variables of these stimuli were also adjusted for a maximal response. Throughout each experiment, recordings were taken from the same muscle cell. This is important because there is a quantitative difference in the size of the responses in different muscle cells. The response size is measured as the difference between the resting membrane potential and the peak of the elicited response. The graphs show the mean of five consecutive responses.

The relationship between the strength of the applied stimulus and the amplitude of the response recorded in muscle cells suggests that the applied stimuli do not evoke single action potentials in neurons and subsequent unitary responses in muscle cells. For both direct and indirect stimulation above a threshold value, increasing the stimulus strength increased the response amplitude until an upper limit is reached. We have not yet investigated the mechanism of this relationship. Possibly the commissure does not carry propagating action potentials, and electrical signals are transmitted passively between the ventral cord and the dorsal cord by decremental spread of current. However, the distances between the nerve cords are large (up to 2 cm) and it would require unusually long space constants of the commissural fibers to provide effective signaling. An alternative explanation is that the stimuli evoke multiple action potentials in the neurons, and the muscle response is related to the number of action potentials generated. If this were true, it should be possible in principle to record

varying numbers of postsynaptic potentials in muscle. In the experiments reported here, intracellular recordings were made from the muscle bellies; the neuromuscular synapses are made not onto the bellies but rather onto the ends of muscle process (arms) that extend from the bellies to the nerve cord. The ends of the arms of different muscle cells are interconnected by electrical synapses. Both these electrical junctions and the spatial separation between the synapses and the recording site might account for the failure to observe increasing numbers of unitary synaptic potentials as the stimulus strength was increased.

RESULTS

Immobilization of Nematodes by AVM. AVM immobilized both parasitic and free-living nematodes. This effect was best demonstrated when 1.5 μg or more was injected into an adult *Ascaris;* the animal became immobilized within a few minutes and did not recover. The immobilized worm was neither in muscular tetanus nor in flaccid paralysis; rather it retained normal muscular rigidity and the capability of contracting when dropped onto a hard surface.

When the free-living nematode *C. elegans* was exposed to AVM, like *Ascaris,* it was immobilized. A mutant of *C. elegans* (E1072) that is resistant to the anthelmintic levamisole gave the same response to AVM—i.e., both wild-type and mutant had a 50% effective dose value of about 0.1 μg/ml for a 10-min incubation at room temperature (Fig. 2). These data suggest that the mode of action of AVM is not related to that of levamisole, which is known to act as an acetylcholine agonist (12).

This conclusion is supported by experiments in which acetylcholine was injected into *Ascaris.* When 50 μg of acetylcholine was injected, the worm hypercontracted and ceased movement. After about 30 min the animal relaxed and resumed movement, presumably because the acetylcholine was digested by endogenous cholinesterase. When *Ascaris* was injected with AVM, a subsequent injection of acetylcholine also produced a hypercontraction and cessation of movement. The animals relaxed after 30 min but, unlike those treated with acetylcholine alone, they remained immobile. These observations suggest that the action of AVM is not related to the action of acetylcholine. The conclusion is further supported by the findings that AVM had no effect on a purified sample of *E. electricus* acetylcholinesterase or on the cholinesterase activity in an extract of *D. viviparus.*

Injecting *Ascaris* with 0.1 mg of acetylcholine, 1 μg of levamisole, or 0.03 mg of neostigmine in 0.1 ml of Ringer's solution caused significant shortening of the worm ($P < 0.05$, t test) compared with injection of the same volume of Ringer's solution. Injection of 0.1 mg of GABA caused a significant

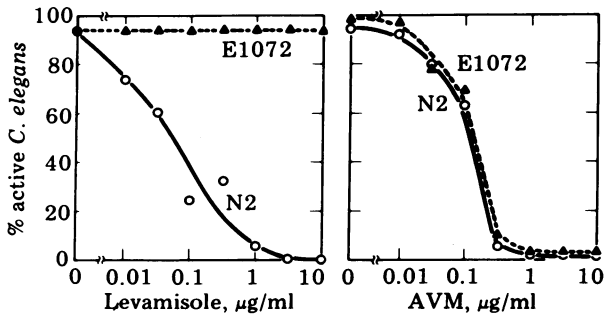

FIG. 2. Effects of levamisole and AVM on the motility of *C. elegans.* Suspensions of *C. elegans* (200–400 adults per ml) were exposed to drugs at designated concentrations. After 10 min, the number of motile worms was counted. N2, wild-type; E1072, levamisole-resistant mutant.

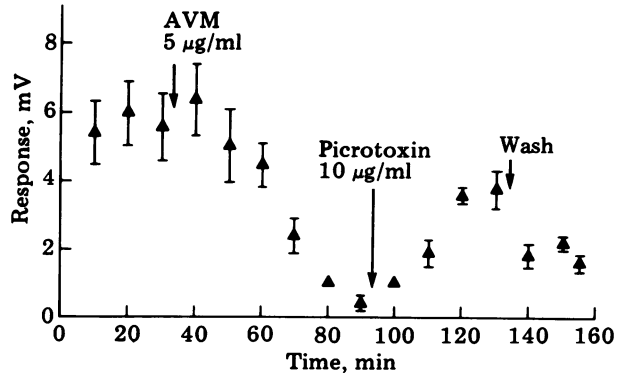

FIG. 3. Responses to indirect stimulation of DE1. Each point represents the mean of five responses. All recordings are from the same dorsal muscle cell. Drugs were added at arrows.

lengthening of the animal. In contrast, there was no significant change in length of the animals injected with 5 μg of AVM.

Effects of AVM and Picrotoxin on the Response to DE1 Motoneuron Stimulation. Stimulating a DE1 motoneuron either directly or indirectly produced a depolarizing response in dorsal muscle. When the commissure of the DE1 motoneuron was severed (Fig. 1*b*, cut 1), these responses no longer could be elicited; therefore, the dorsal response is indeed mediated by the DE1 motoneuron (11). The response to indirect stimulation also could be eliminated by cutting the ventral cord between the indirect stimulating electrode and the DE1 ventral process (Fig. 1*b*, cut 2). This shows that "indirect stimulation" does not activate the DE1 neuron through current spread from the indirect stimulating electrode.

The response to indirect stimulation of DE1 was diminished upon addition of AVM at 5 μg/ml (Fig. 3). The response declined gradually and often was completely blocked after 30–50 min. Picrotoxin reversed this effect of AVM, causing the response to indirect stimulation to increase. We were unable to reverse the effects of AVM by washing with drug-free Ringer's solution; however, we could wash out picrotoxin's reversal of the effect of AVM. When we washed a preparation that contained both AVM and picrotoxin with drug-free Ringer's solution, responses to indirect stimulation decreased again (Fig. 3).

AVM sometimes caused a decrease in the direct response; however, it always caused a much greater decrease in the indirect response (Fig. 4). Sometimes, as in Fig. 5, there was a gradual decline in the responses that was unrelated to the presence of AVM; often there was no such decline. Picrotoxin raised the level of the indirect response back to that of the direct response. The level of the direct response was not changed by picrotoxin even if it had decreased after addition of AVM (Fig. 4). Picrotoxin alone had no effect on the directly or indirectly stimulated response.

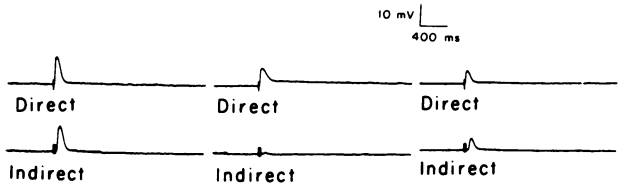

FIG. 4. Effect of AVM and picrotoxin on DE1 responses. All recordings were from the same dorsal muscle cell. (*Left*) Control responses. (*Middle*) AVM (30 min; 5 μg/ml) greatly decreased the indirect response; the direct response was only slightly diminished. (*Right*) Picrotoxin (20 min; 10 μg/ml) partially restored the indirect response.

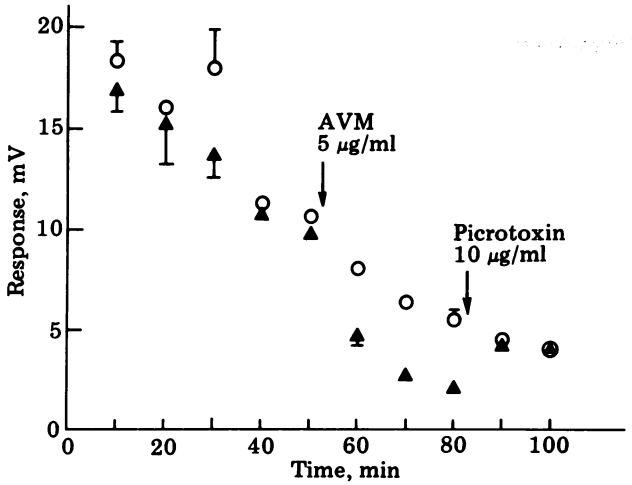

FIG. 5. Effects of AVM and picrotoxin on direct (○) and indirect (▲) responses of DE1. SEM bars are shown only on one side of each point and are omitted where they underlie symbols.

These results indicate that the major effect of AVM on the excitatory motoneurons is via the pathway in the ventral nerve cord by which the excitatory neurons are activated.

Effect of AVM and Picrotoxin on the VI Motoneuron Response. Stimulation of the dorsal process of the VI motoneuron elicited a hyperpolarization in the ventral musculature. The amplitude of this hyperpolarization, typically about 2–4 mV, varied from muscle cell to muscle cell and from preparation to preparation; however, it remained relatively constant over time for an individual muscle cell. Picrotoxin in concentrations as high as 100 μg/ml (most experiments used 10–20 μg/ml) did not block the hyperpolarization due to VI stimulation. Addition of GABA to the bath also caused a hyperpolarization whether or not picrotoxin was present.

When AVM was applied to a preparation in which the VI motoneuron was continuously stimulated at a rate of once per 4 sec, the hyperpolarizing response was reduced (Fig. 6). This decline only was seen in the presence of AVM and was not reversed by picrotoxin. The resting potential of muscle cells was unaffected by AVM.

Lack of Effect of AVM on Nematode Metabolism. *D. viviparus* generates lactic acid under aerobic conditions (13). Twelve adult worms in *Ascaris* Ringer's solution at 37°C excreted, on the average, 10 mg of lactic acid per hr; the rate was linear for the first 8 hr of incubation. AVM up to 0.1 mg/ml exerted no effect on the rate of lactate production.

T. colubriformis carries out CO_2 fixation in its anaerobic metabolism (13). When incubated in *Ascaris* Ringer's solution under N_2 at 37°C, the worms incorporated $^{14}CO_2$ at a linear rate for the initial 8 hr; 25% of incorporated radioactivity was present as succinate and 40% as glutamate and aspartate. Neither the rate of incorporation nor the distribution of radioactivities was affected by AVM up to 0.1 mg/ml.

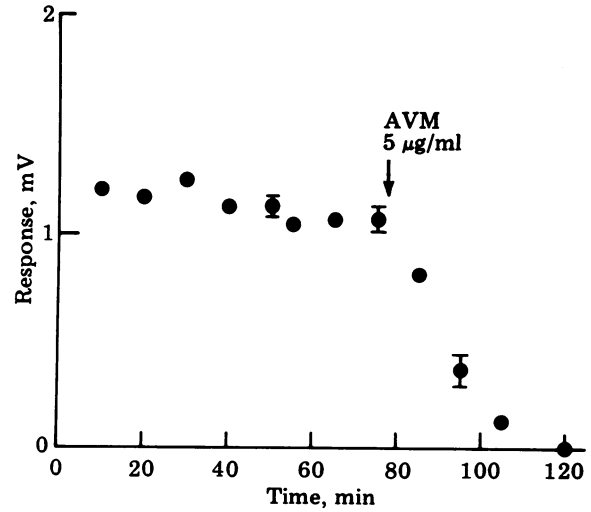

FIG. 6. Effect of AVM on the responses to direct stimulation of VI.

DISCUSSION

In our experiments on the electrophysiology of *Ascaris*, the dorsal and ventral nerve cords remained connected by the commissures of only two neurons, DE1 and VI; all the other connections between the dorsal and ventral cords were severed. By both anatomical and physiological methods it has been shown that the DE1 motoneuron has output to muscle in the dorsal but not the ventral half and that the VI neuron has output to muscle only in the ventral half (11). Therefore, by stimulating ventrally while recording from dorsal muscle, we can selectively monitor the output of the DE1 motoneuron; the only other intact motoneuron is the VI which has no dorsal output to muscle. Similarly, by stimulating dorsally and recording ventrally, we can selectively monitor the output of the VI motoneuron which has ventral, but no dorsal neuromuscular synapses.

The stimulating electrodes were placed over the entire nerve cord and probably stimulated many of the neural processes that underlie them. It is reasonable to assume that current flow from the direct stimulating electrode activates the DE1 motoneuron directly; however, other motoneurons or interneurons in this part of the cord might also be activating or inhibiting DE1 synaptically. The experiment in which the cord was cut between the indirect stimulating electrode and the DE1 ventral process demonstrates that the DE1 dendrite is not activated by current spread from the indirect stimulating electrode but rather that an intact neural pathway between the stimulating electrode and the dendrite is necessary. We believe that the DE1 neuron is activated by one or more of the five large interneurons that run along the ventral cord because it has been shown by electron microscopy that: (*i*) many synapses exist between these interneurons and the DE1 dendrite, and (*ii*) the processes of other excitatory motoneurons are not seen to synapse onto DE1 in the ventral cord (unpublished data). Furthermore, the indirect stimulation of DE1 is curare-sensitive (unpublished data), which is also consistent with the idea that the DE1 dendrite is activated synaptically.

AVM causes the response to indirect stimulation of the DE1 motoneuron to decrease relative to the direct response. There are several possible explanations. (*i*) AVM might affect conduction down an excitatory interneuron so that the electrical signal generated at the stimulating electrode either fails or is highly attenuated by the time it reaches the synapse to the motoneuron. (*ii*) AVM might inhibit the synapse between the excitatory interneuron and the DE1 motoneuron. For example, AVM might be acting as an antagonist to the excitatory transmitter or inhibit its release at this synapse. (*iii*) AVM might inhibit the dendrite of the DE1 motoneuron, either by mimicking an inhibitory transmitter or by causing the release of an inhibitory substance from another neuron.

We believe that AVM acts by affecting inhibitory synaptic mechanisms, in which GABA is the probable chemical transmitter (14). We have shown that AVM blocks the hyperpolarizing response of muscle to stimulation of the VI motoneuron. We have also found (unpublished data) that if piperazine, which is an inhibitory agonist in *Ascaris* (14), or muscimol, a well-known GABA agonist, is applied to the ventral nerve cord,

the indirect response of DE1 is eliminated but the direct response is unaffected. Like the AVM-induced blockage of indirect stimulation of DE1, the effects of both of these compounds are reversed by picrotoxin.

In other systems, the action of AVM has been shown to be closely related to GABAnergic systems. At the lobster neuromuscular junction, AVM has a GABA-like effect, either by causing release of GABA from presynaptic nerve terminals or by acting as a GABA agonist (6). AVM has been shown to cause release of GABA from mammalian synaptosomes (7).

The most economical hypothesis about the action of AVM in *Ascaris*, therefore, is that it simulates inhibitory neuronal input, either by acting as a GABA agonist or by stimulating GABA release from presynaptic inhibitory terminals. Either mechanism would require the presence of GABA receptors on the excitatory interneuron or the DE1 dendrite. If these GABA receptors were on the excitatory interneuron, then AVM should lead to inhibition of the excitatory interneuron and decrease its ability to propagate electrical signals. Because the source of synaptic input onto interneurons is almost exclusively from other interneurons (unpublished data), this would imply that some of the interneurons are inhibitory, although at present there is no direct evidence that this is the case. If AVM or the released inhibitory transmitter (again released from an inhibitory interneuron) acts directly on the DE1 dendrite, it might appear that the differential effect of AVM on indirect and direct responses is not what would be expected. However, anatomical studies show that the input from each interneuron is distributed at several sites along the length of the DE1 dendrite. Presumably there is integration of the excitatory activity from these sites; by reducing the input resistance of the dendrite, inhibition would therefore reduce the efficacy of the excitatory synaptic input. For direct stimulation the effect of inhibiting the dendrite might be minimal, especially because the extracellular stimulating electrode was located near the origin of the commissure that carries the electrical signal to the dorsal musculature and the stimulus was supramaximal.

In order to test these ideas further, it will be necessary to identify inhibitory interneurons, to determine whether they use GABA as a chemical transmitter, and to localize GABA receptors by electrophysiological or morphological techniques.

At present we do not have a complete understanding of the way that the locomotory movements of *Ascaris* are coordinated. However, because the inhibitory motoneurons receive their sole input from the axons of excitatory motoneurons, clearly the control of the excitatory motoneurons is central in the motor program (15). Anatomical studies have shown that the dendrites of the excitatory motoneurons receive their synaptic input almost exclusively from interneurons. It is therefore to be expected that any disruption of the signaling between interneurons and motoneurons, such as that caused by AVM, would lead to paralysis of the animal.

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