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BEHAVIORAL EFFECTS OF IVERMECTIN IN A FRESHWATER OLIGOCHAETE, *LUMBRICULUS VARIEGATUS*

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Abstract—Ivermectin is a potent antiparasitic drug against nematode and arthropod parasites. In this study, we examined the lethal and sublethal effects of ivermectin in a freshwater oligochaete, *Lumbriculus variegatus.* The median lethal concentration (LC50) at 72 h after ivermectin exposure was 560 nM. Sublethal endpoints focused on several stimulus-evoked locomotor behaviors: escape reflexes controlled by giant interneuron pathways, swimming and reversal, and crawling. Swimming, reversal, and crawling are controlled by nongiant interneuron pathways. Ivermectin inhibited swimming, reversal, crawling frequency, and crawling speed in a time- and concentration-dependent manner with a mean inhibitory concentration (IC50) at 3 h of 1.1, 16, 91, and 51nM, respectively. Ivermectin at 0.3 nM also significantly decreased the frequency of helical swimming waves. Picrotoxin, a Cl^- channel blocker, antagonized the ivermectin-induced decrease in swimming frequency, crawling frequency, and crawling speed. There were no adverse effects on escape reflex 3 h after exposure to 300 nM ivermectin. Electrophysiological recordings showed that ivermectin had no effects on the conduction velocity of giant fiber systems. The results indicated that locomotor behaviors controlled by nongiant locomotor pathways were more sensitive to ivermectin than pathways controlled by giant interneurons and that Cl^- channels may be involved in mediating ivermectin's inhibitory effects.

Keywords—Ivermectin Sublethal Behavior Locomotion Invertebrate

INTRODUCTION

Ivermectin (22, 23-dihydroavermectin B_{1a}), a semisynthetic avermectin analog, is a potent anthelmintic and insecticide used on nematode and arthropod parasites. It is widely used to improve the health care of domestic animals [1,2] and humans [3,4]. Although ivermectin's mode of action is not fully understood, it is generally believed that ivermectin reduces excitability of muscle or nerves through the opening of Cl^- channels [5–9].

Environmental effects and fates of ivermectin have also been studied [10–14]. In the vast majority of these studies, mortality has been the endpoint. *Daphnia magna* was particularly sensitive to ivermectin (48-h LC50 0.025 ppb). Bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*) were less sensitive (48-h LC50 4.8 and 3.0 ppb, respectively). Earthworms (*Eisenia foetida*) were relatively insensitive to ivermectin (28-d LD50 315 ppm in soil) [11]. Without analysis of sublethal endpoints (e.g., effects on growth, development, fecundity, morphology, behavior, or physiology), we cannot fully understand the complex biological actions or predict ecological impacts of an environmental toxicant. This is especially true for a chemical like ivermectin, which exerts antiparasitic effects not by instantly killing the target organisms but by reducing their motor activities so that the parasites are excluded from the host [15].

Studies have evaluated the sublethal effects of ivermectin on development and reproduction of nontarget organisms, mostly in dung-dwelling insect populations that may be threatened by drug residues in the manure from ivermectin-treated animals [11]. Ivermectin residues inhibited larval development of bushfly, *Musca vetustissima* and *Musca domestica* [16–18]. Adult dung beetles (*Copris hispanus, Bubas bubalus,* or *Onthophagus binodis*) survived exposure to ivermectin residue in dung, but the rate of oviposition was reduced [16,19,20].

Locomotor capabilities are logical focal points for studies of sublethal effects of ivermectin because the most predominant effects shown in target organisms are reduced motor activities [15]. However, effects of ivermectin on motor activity on nontarget organisms are poorly understood. In this study, we examined sublethal effects of ivermectin on locomotor behaviors of a nontarget invertebrate, *Lumbriculus variegatus.*

Features that make *L. variegatus* especially suitable for this study include their ubiquitous inhabitation in North America and Europe and their introduction into Africa, Australia, and New Zealand [21]; their freshwater benthic habitat, in which they are vulnerable to the possible runoff of ivermectin within eroding sediments; their ease in laboratory rearing, maintenance, and handling; their defined patterns of locomotor behaviors, namely, helical swimming, body reversal [22], and crawling [23]; the presence of giant nerve fibers (interneurons) that mediate rapid escape responses [24–26]; and the capability of noninvasive electrophysiological testing of escape reflex function [24,27,28].

Locomotor behaviors in *L. variegatus* are context specific. When the worm's tail is extended above the sediments, it responds to the tactile stimulation or shadow by a rapid withdrawal (escape response). On wet surfaces or in confined spaces underwater (e.g., in muddy sediments or in between decaying leaves), the worm crawls forward or backward when touched in the tail or head region, respectively. In open spaces underwater, however, tail stimulation evokes helical swimming, while head stimulation evokes body reversal [22]. These locomotor behaviors are highly stereotyped, thus making them ideal for sublethal toxicological tests.

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Our specific objectives were as follows: determine lethal concentrations of ivermectin to *L. variegatus;* evaluate sublethal effects of ivermectin on locomotor behaviors (swimming, reversal, and crawling); evaluate possible involvement of Cl^- channels using picrotoxin, a Cl^- channel blocker; and evaluate electrophysiological effects of ivermectin on giant nerve fiber pathways.

MATERIALS AND METHODS

Animal culture and selection

Lumbriculus variegatus were reared in the laboratory at 22 ± 1°C in aerated aquaria containing pieces of brown paper towel [28]. Worms were fed three to five times per week with fish food.

Medium-size worms (~4–5 cm long) were removed from rearing tanks 12 to 24 h prior to testing and placed in petri dishes containing distilled water to allow clearance of gut contents. Worms were visually screened for uniformity in segmentation pattern. Worms showing recent segment regeneration or any obvious morphological defects were not used.

Chemical preparation

The following chemicals were used: ivermectin stock solution (11.5 mM in 40% glycerol formal and 60% propylene glycol, Merck AgVet, Rahway, NJ, USA) and picrotoxin (Sigma Chemical, St. Louis, MO, USA). Ivermectin solutions were prepared by diluting ivermectin stock solution in distilled water. Additional propylene glycol was added to all solutions with lower ivermectin concentrations so that the concentration of solvent in all solutions was the same. Control solutions also had the same concentration of solvent as the ivermectin solutions. For example, in swimming and reversal ability test, the highest concentration of ivermectin was 300 nM, so the concentration of solvent was 0.0026% (v/v). Additional 2.60, 2.60, 2.57, and 2.34 μl of propylene glycol were added to solvent-only, 0.3-, 3-, and 30-nM ivermectin solutions (100 ml total volume), respectively. In ivermectin and picrotoxin antagonism experiments, picrotoxin was dissolved in distilled water. After worms were exposed in the picrotoxin solutions for 60 min, ivermectin and/or propylene glycol were added to the solutions. Concentration levels of ivermectin and/or picrotoxin were determined according to preliminary range-finding experiments. All concentrations reported are nominal; no analytical procedures were performed to verify the actual concentrations of the chemicals. However, all aqueous solutions were prepared immediately before the experiments.

Experimental design

Exposure was carried out in covered glass petri dishes (9 cm in diameter, 2 cm in depth) with one worm per container of 100 ml (for swimming and reversal tests) or 50 ml (for other tests) solution. Individual worms were randomly assigned to the concentration levels. Each level was replicated 10 to 21 times. In swimming and reversal tests, worms were examined directly in the exposure dishes. In other tests (swimming frequency, crawling, and electrophysiology), worms were quickly rinsed twice in distilled water and temporarily removed from the exposure dishes for behavioral or electrophysiological testing.

Lethal concentration

Fifty worms were randomly assigned to five concentration levels (180, 320, 560, 1,000, and 1,800 nM), with 10 worms per level. Determination of lethality was made after 24 and 72 h exposure duration. Mortality was indicated by decomposition of the worms. In a separate experiment, 10 worms were exposed to a very high concentration (2,400 nM) of ivermectin for a relatively short duration (8 h) and then transferred to distilled water to determine the possibility of recovery.

Behavioral testing

Swimming and reversal. Helical swimming and body reversal behaviors in *L. variegatus* were studied as previously described [22]. The worms' ability to initiate swimming and/or reversal episodes was tested following 0, 1, 3, and 8 h of exposure (concentration levels: solvent, 0.3, 3, 30, and 300 nM ivermectin). In each test, a worm was touched 10 times with a rubber probe alternately at its anterior or posterior end to evoke reversal and swimming, respectively. The interval between successive touches was 3 to 5 s. A response to a touch stimulus was scored as successful only when the worm showed stereotypic patterns of swimming or reversal movements.

Swimming frequency and pattern. To quantify possible effects of ivermectin on swimming frequency, a worm was placed in the middle of a plastic petri dish (14 cm in diameter, 2.5 cm in depth) containing 200 ml of distilled water. Swimming responses were evoked twice by tactile stimulation to the posterior end of the worm using a rubber probe [22]. The worm was allowed to rest about 2 min after it was moved into the dish and between the two trials. The process was recorded on VHS videotape using a videocassette recorder (Mitsubishi, model HS-U650, Tokyo, Japan) connected to a camcorder (LXI, model 934.53796290, Irving, TX, USA) and replayed frame by frame on a video monitor (NEC, model XM-2950, Nippon Electric Company, Melville, NY, USA) after testing to examine the swimming pattern and frequency (number of helical body waves produced per second). Each worm's responses were measured before and at a selected time after exposure (0 and 3 h in the ivermectin-alone and 0 and 1.5 h in the ivermectin–picrotoxin antagonism experiment). The ratio of the mean frequency after exposure to the mean frequency before exposure was defined as relative swimming frequency for each worm. When exposed to higher concentrations of ivermectin, some worms failed to swim in one trial or both trials. In such cases, only successful trials were used to calculate relative swimming frequency. If the worm failed twice, the failures were recorded in a separate category. These failures were not used for calculation of mean swimming frequency. Five concentration levels were used in ivermectin-alone experiments (0, 0.03, 0.3, 3, and 30 nM). Twelve concentration levels were used in ivermectin–picrotoxin antagonism experiments (all combinations of two levels of ivermectin [0 and 30 nM] and six levels of picrotoxin [0, 1, 10, 100, 1,000, and 10,000 nM]).

Crawling. In the crawling test, a worm was placed next to a smooth strip of Plexiglas (180 × 40 × 6 mm) that rested on a piece of thoroughly wetted filter paper (Whatman No. 1, Bangkok, Thailand). Any excess water was removed, thus confining the worm within the surface tension of a narrow band of water between the Plexiglas and paper. A straight rubber band (5 mm long, 0.5 mm in diameter, attached to a wooden applicator stick) was used to brush the worm's tail so that the worm would crawl forward in a straight line along the Plexiglas. The frequency of brushing was 3.6 ± 0.1 strokes/s (n = 20) as determined from videotape replay. This stimulation

lasted 10 to 15 s, or until the worm had crawled 4 to 6 cm. The same procedure was repeated once, and the worm was allowed to rest for about 1 min between trials. Crawling behavior was recorded on videotape and later replayed, frame by frame, to measure the crawling speed (distance moved per second) and frequency (number of peristaltic waves of contraction produced per second). Each trial consisted of one to three episodes of continuous crawling movements. Episodes that had relatively constant crawling frequency were used to calculate speed and frequency. Only the episode with the highest crawling speed was used for analysis. Each parameter was measured twice, once before (0 min) and once after the exposure (15, 30, 60, 120, or 180 min in ivermectin-alone experiments, 90 min [60-min picrotoxin preexposure + 30-min ivermectin exposure] in ivermectin–picrotoxin antagonism experiments). Each of these values represents the highest value obtained from one to six episodes performed by each worm. Relative crawling frequency and relative crawling speed were defined as previously described for relative swimming frequency. In ivermectin-alone experiments, five levels of ivermectin were used for 180-min exposure (0, 10, 30, 100, and 300 nM), while three levels were used for other durations of exposure (0, 30, and 300 nM). Twelve concentration levels were used in ivermectin–picrotoxin antagonism experiments (all combinations of two levels of ivermectin [0 and 300 nM] and six levels of picrotoxin [0, 1, 3, 10, 30, and 100 μM]).

Electrophysiological testing

Techniques for noninvasive electrophysiological recording were used as previously described [24,27,28]. Briefly, a worm was placed next to a smooth strip of Plexiglas (4 × 1 cm) on a printed circuit board recording grid moistened with distilled water. Distilled water, rather than spring water, is routinely used for recording giant fiber electrical signals [24,27,28]. Excess water was removed, thus trapping the worm in surface tension along a narrow band of water between the Plexiglas and electrode grid. The worms' medial and lateral giant fiber (MGF and LGF) systems were activated by tactile stimulation to the anterior and posterior ends of the worms, respectively. Evoked spikes were detected by two pairs of recording electrodes. Signals were amplified, filtered, and displayed as two channels on a digital oscilloscope (TENMA, model 72-915 20 MHz, Premier Farnell, Leeds, UK). Giant fiber conduction velocity was measured at a midbody location over a 10-mm conduction distance. To obtain velocity, conduction distance was divided by conduction time, as indicated on the oscilloscope screen by the peak-to-peak interval between spikes in the two recording channels. Each worm was measured before and after the exposure (0 and 3 h). Mean velocity (five measurements per worm) was then converted to relative conduction velocity, which was defined as the ratio of the mean velocity at any time after exposure compared to the mean velocity in the same worm before exposure. Therefore, by definition, the relative velocity before exposure in each worm was 1.0.

Data analysis

Mean lethal concentration (LC50) and 95% confidence interval were calculated using the method described by Well [29]. Mean inhibitory concentrations (IC50) were calculated using the computer program Pharmcalc.Bas (Microsoft®, Seattle, WA, USA).

In all cases, including figures, parametric data were expressed as means ± standard error of the mean (SEM) and analyzed by analysis of variance (ANOVA). The *F* value was used to establish significance for the treatment effect. Then the least-significant-difference test was used to determine significance of each concentration level. In picrotoxin and ivermectin antagonism experiments, data were analyzed using the SAS® Proc General Linear Means (GLM) procedure (Cary, NC, USA). Significance of interaction between ivermectin and picrotoxin was established using two-way factorial analysis. The significance of each concentration level of picrotoxin was then evaluated using simple effect comparisons; that is, the ivermectin-induced effect at a given level of picrotoxin was compared against the effect without picrotoxin. The χ^2 test was used to evaluate significance of the nonparametric swimming pattern change. The significance level was set at $p < 0.05$.

Table 1. Effects of ivermectin on locomotor behaviors in *Lumbriculus variegatus*

Behavioral endpoints	Swimming frequency	Swimming ability	Reversal ability	Crawling speed	Crawling frequency	Escape reflex
IC50 (nM)[a]	0.3[b]	1.1	16	51	91	No effect

[a] Mean inhibitory concentration after 3 h of exposure to ivermectin.
[b] Least-significant-effect concentration.

RESULTS

Lethal effect

Ivermectin concentrations of 560 nM or greater were lethal to worms. The LC50 at 72 h post–ivermectin exposure was 560 nM (95% confidence interval: 440–720 nM). A characteristic of ivermectin's lethal effect in *L. variegatus* was that the concentration range for 0 to 90% death was narrow, representing only a threefold difference. It was also evident that onset of ivermectin-induced mortality in *L. variegatus* was delayed. At a concentration of 1,000 and 1,800 nM, only one and three out of 10 treated worms, respectively, died within 24 h of exposure. Most worms (eight and six, respectively) died between 24 and 72 h after exposure. In another experiment, 10 worms were treated with 2,400 nM (extrapolated 72 h LC_{99}) ivermectin for 8 h and then transferred to distilled water; all of them recovered. This is additional evidence for the delay in ivermectin-induced mortality.

Sublethal effects

Our subsequent experiments focused on ivermectin's sublethal effects at concentrations of 0.03 to 300 nM. During the first hour after exposure to 300 nM ivermectin, worms became flaccidly paralyzed. The worms' bodies became thinner and longer. Spontaneous movement was totally inhibited, as were swimming, reversal, and crawling. In worms exposed to lower concentrations of ivermectin, the effects were similar but less extreme and more delayed in onset. In contrast to the ivermectin effects on slow locomotor movements, rapid escape reflexes appeared less affected by these exposures (see last section of Results). Table 1 summarizes ivermectin's sublethal effects on various behavioral endpoints. Details are described in the following sections.

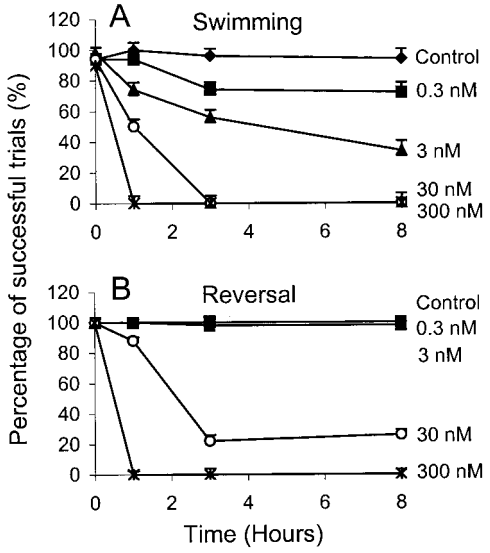

Fig. 1. Time- and concentration-dependent inhibition of ivermectin on helical swimming and body reversal behaviors in *Lumbriculus variegatus*. Individual worms were tested for their ability to perform touch-evoked swimming and reversal behaviors before and at specified time points during ivermectin exposure (five trials per test). Swimming and reversal were visually categorized as success or failure. (**A**) Effect of ivermectin on swimming during 8 h continuous exposure. (**B**) Effect of ivermectin on reversal during 8 h exposure. Values are mean ± standard error of the mean (SEM) (n = 10).

Effect of ivermectin on locomotor behaviors controlled by nongiant interneuron pathways

Swimming and reversal. Ivermectin inhibited helical swimming and body reversal behaviors in a time- and concentration-dependent manner (Fig. 1). There were no observable behavioral changes within 10 min of exposure to 300 nM ivermectin. However, within 60 min of ivermectin administration, swimming and reversal were abolished. Ivermectin at 30 nM also abolished swimming (within 3 h) but not reversal. The IC50 at 3 h for swimming and reversal were 1.1 and 16 nM, respectively (Table 1).

Swimming frequency and swimming pattern. Normal swimming movements consist of a series of alternating left- and right-handed, rapid, and rhythmic waves of helical body bending. Each wave rapidly progresses from head to tail, thus propelling the worm forward [22]. The swimming frequency in untreated worms was consistent at 10.7 ± 0.1 Hz (n = 80). Ivermectin at 0.3 nM or higher significantly decreased the swimming frequency 3 h after exposure (Fig. 2). At a concentration of 30 nM, six out of 16 worms failed to swim. There were qualitative changes in the basic pattern of swimming in another three worms. In these worms, helical waves appeared to initiate from the middle of the body rather than from the anterior end. In the remaining seven worms, there was no qualitative change in swimming pattern, but the swimming frequency was further decreased (Fig. 2).

Picrotoxin, a Cl^- channel blocker, was used to assess its possible influence on ivermectin-induced (30 nM) decreases in swimming frequency and changes in swimming pattern. Picrotoxin alone (1–10,000 nM) had no effect on swimming frequency (Fig. 3A). Ivermectin decreased swimming frequency by 4% in picrotoxin 0-nM groups (the difference between the first hatched and open bars, $p < 0.05$). The differences were also significant at picrotoxin 1- and 10-nM groups, but not 100-, 1,000-, and 10,000-nM groups.

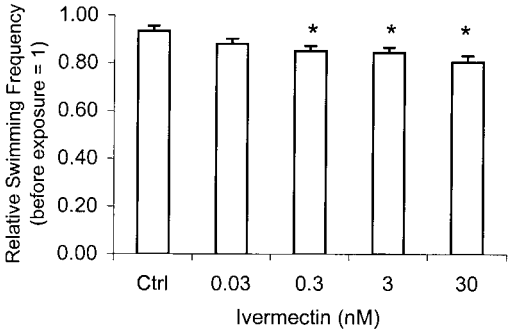

Fig. 2. Inhibitory effect of ivermectin on swimming frequency in *Lumbriculus variegatus*. Swimming frequency (number of helical body waves per second) was measured using video analysis. Each worm was tested before and 3 h after ivermectin exposure. In the 30-nM group, only 10 of 16 worms could swim after 3 h of ivermectin exposure. Values are mean ± standard error of the mean (SEM) (n = 16). * $p < 0.05$ compared to control group.

Picrotoxin also antagonized the ivermectin-induced changes in the qualitative pattern of swimming (Table 2). Picrotoxin alone (1–10,000 nM) did not cause significant changes. Ivermectin at 30 nM caused abnormal swimming pattern in six of 20 worms. Picrotoxin at 10 and 100 nM significantly reversed the effect of ivermectin. However, higher concentrations of

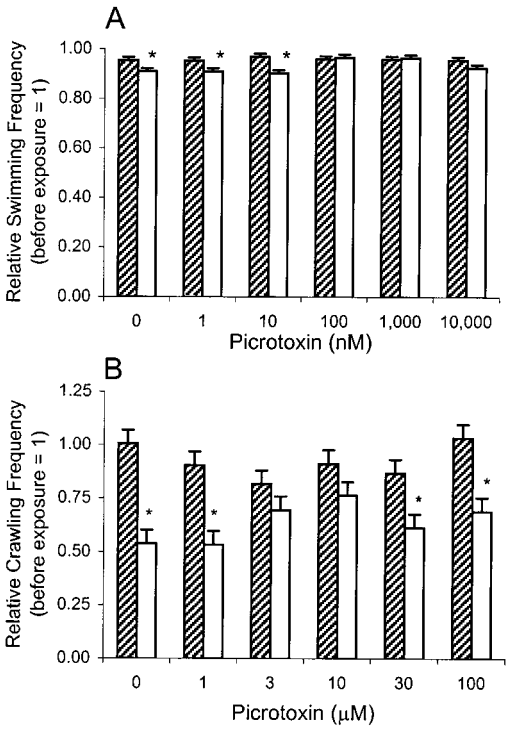

Fig. 3. Antagonism of picrotoxin on the inhibitory effects of ivermectin in the locomotor behaviors in *Lumbriculus variegatus*. Swimming frequency (number of helical body waves per second) and crawling frequency (number of peristaltic waves of contraction per second) were measured using video analysis. The worms were pretreated with picrotoxin for 60 min before ivermectin administration for 30 min. Each worm was tested twice, before picrotoxin pretreatment and 30 min after ivermectin administration. (**A**) Effect of picrotoxin on ivermectin-induced decrease in swimming frequency (n = 20). ▨: solvent, □: ivermectin, 30 nM. (**B**) Effect of picrotoxin on ivermectin-induced decrease in crawling frequency (n = 10). * $p < 0.05$, compared to solvent-only group at the corresponding picrotoxin dose level. ▨: solvent, □: ivermectin, 300 nM. Values are mean ± standard error of the mean (SEM).

Table 2. Antagonism by picrotoxin of ivermectin-induced change of swimming pattern in *Lumbriculus variegatus*

Ivermectin	Picrotoxin (nM)					
	0	1	10	100	1,000	10,000
Solvent	0/20[a]	0/20	0/20	0/20	1/20	1/20
30 nM	6/20	3/20	0/20[b]	1/20[b]	2/20	7/20

[a] Number of worms out of 20 that showed abnormal swimming patterns.
[b] $p < 0.05$, compared to the 30-nM ivermectin, 0-nM picrotoxin group.

picrotoxin (1,000 and 10,000 nM) did not significantly antagonize the pattern changes.

Crawling. Forward crawling movements consist of a series of rhythmic peristaltic waves of body contraction. Each wave begins at the anterior end of the worm and progresses toward the tail. In contrast to swimming frequency, there was a great variation in wave frequency and forward velocity of crawling. We attempted to reduce this variation by using only the most vigorous crawling episodes for each worm. We found that one way to minimize the variation in crawling frequency and speed was to stimulate the worms to crawl as fast as possible. To do this, we repetitively brushed the worms' tails at a frequency of three or four strokes per second.

In untreated worms, the crawling frequency and crawling speed were 1.21 ± 0.02 Hz and 6.1 ± 0.1 mm/s, respectively ($n = 127$). Ivermectin decreased crawling frequency in a time- and concentration-dependent manner (Fig. 4A, 4B). Crawling was totally inhibited after 3 h of exposure to 300 nM of ivermectin. The IC50 at 3 h for crawling frequency was 91 nM (Table 1).

Picrotoxin antagonized the ivermectin-induced decrease in crawling frequency. Picrotoxin alone at all concentrations studied (1–100 μM) did not change crawling frequency (Fig. 3B). Ivermectin at 300 nM significantly decreased crawling frequency by 47% in the picrotoxin 0-nM group. Picrotoxin (3 and 10 μM) significantly antagonized the effect of ivermectin, as indicated by the lack of significant differences between the ivermectin and solvent groups. However, picrotoxin at 1, 30, and 100 μM failed to do so (Fig. 3B).

Ivermectin's inhibitory effect on crawling speed was similar to its effect on crawling frequency. The IC50 for crawling speed at 3 h was 51 nM (Table 1). Ivermectin-induced decrease in crawling speed was significantly reduced from 52 to 19% by 10 μM picrotoxin.

Effect of ivermectin on behavior controlled by giant interneuron pathways

The worms retained escape reflex function throughout the 3 h of exposure to 300 nM ivermectin (Table 1). That is, while crawling on substrate, worms were capable of rapidly withdrawing head or tail in response to tactile stimulation. However, the escape response was not followed by any slower locomotor movements (swimming, reversal, or crawling), which usually occurred immediately after escape responses in normal worms. In addition, while normal worms had little difficulty in rapidly withdrawing in response to repeated tactile stimulation, the escape withdrawal in treated worms was not visually detectable after four or five repeated stimuli. When the worms were allowed to rest for 1 or 2 min, their escape reflex appeared recovered.

Median giant fiber (MGF) and lateral giant fiber (LGF) spiking, recorded noninvasively, was used as an indicator of the function of giant interneuron pathways. In untreated worms, conduction velocity of MGF and LGF was 8.8 ± 0.1 m/s and 6.4 ± 0.1 m/s ($n = 60$), respectively. With ivermectin up to 300 nM, there was no statistically significant change in either MGF or LGF conduction velocity. There was also no apparent hypersensitivity or hyposensitivity of giant fibers to tactile stimulation.

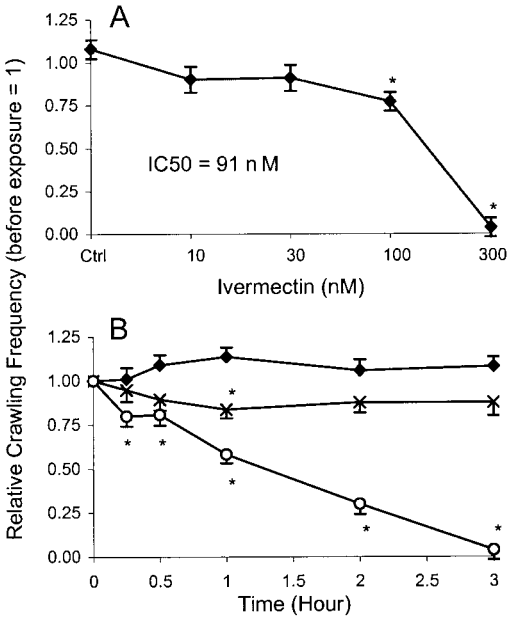

Fig. 4. Time- and concentration-dependent inhibition of ivermectin on crawling frequency in *Lumbriculus variegatus*. Crawling frequency (number of peristaltic waves of contraction per second) was measured using video analysis. Each worm was tested twice, before and after a certain duration of ivermectin exposure. (**A**) Effect of ivermectin on crawling frequency 3 h after exposure ($n = 21$). * $p < 0.05$, compared to controls. (**B**) Effect of ivermectin on crawling frequency during 3 h of exposure ($n = 10$–21). * $p < 0.05$, compared to the solvent-only groups at the corresponding time point. —◆—: solvent, —X—: 30 nM, —○—: 300 nM. Values are mean ± standard error of the mean (SEM).

DISCUSSION

Some environmental fate and effect studies on nontarget organisms have been carried out in the development of ivermectin as an antiparasitic agent [11,30]. The lethal level of ivermectin has been determined in some nontarget organisms. Among them, *D. magna* was most sensitive, with 48-h LC50 of 0.025 ppb [~0.03 nM) [11]. Fish were less sensitive (48-h LC50s for bluegill, *Lepomis macrochirus*, and rainbow trout, *Oncorhynchus mykiss*, were 4.8 ppb [~5.5 nM] and 3.0 ppb [~3.4 nM], respectively), and earthworms (*Eisenia foetida*, 28-d LD50 315 ppm in soil) were relatively insensitive to ivermectin [11]. Our results showed that *L. variegatus* was less sensitive to ivermectin (72-h LC50 of 560 nM ~490 ppb) than *D. magna* and fish. The differences between the exposure method (aqueous solution vs soil) and time scale (3 d vs 28 d) makes the comparison of toxicity in the two oligochaetes difficult.

Although the most predominant effects shown in target organisms are reduced motor activities, there are few studies in the literature examining sublethal effects of ivermectin on nontarget organisms. Studies of sublethal effects of ivermectin on nontarget organisms, mostly concerning development and

reproduction in dung-dwelling insect populations, have been reviewed by Halley et al. [11]. The present study was the first attempt to examine the effects of ivermectin on locomotor behaviors of a nontarget invertebrate, *L. variegatus.*

Our results showed that ivermectin impaired the worms' locomotor behaviors. Behavioral endpoints were much more sensitive than the survival endpoint. The LC50 at 72 h was 560 nM, while 3-h IC50s for swimming, reversal, crawling speed, and crawling frequency were 1.1, 16, 51, and 91 nM, respectively. At 0.3 nM (lower than LC50 by more than three orders of magnitude), ivermectin decreased swimming frequency significantly. The differences in sensitivity to ivermectin indicated that motor behaviors in *L. variegatus* are probably independent of each other. Therefore, it is important to have multiple endpoint measurements to obtain an overall picture of the effect of ivermectin to invertebrates. Our results demonstrated that *L. variegatus* is a sensitive model for assessing sublethal locomotor behavioral effects of environmental toxicants.

It was noted that the onset of paralytic effect of ivermectin in *L. variegatus* was rather delayed. Within 10 min of exposure to 300 nM ivermectin, there was no observable behavioral change. This is in sharp contrast to some other neuroactive chemicals (e.g., 4-aminopyridine, cadmium chloride, carbofuran, chloroform, and diazinon) that caused behavioral changes in *L. variegatus* almost immediately on exposure ($<$1 min) [28]. Our results indicated that the onset of ivermectin-induced mortality in *L. variegatus* was also delayed. A possible explanation for the slow actions of ivermectin is the availability of the chemical to the worms. Although ivermectin is lipophilic, it is a relatively large molecule (mol wt $\sim$870) compared to 4-aminopyridine, cadmium chloride, carbofuran, chloroform, and diazinon (mol wt 94, 183, 221, 119, and 304, respectively). Therefore, it may be more difficult for ivermectin to penetrate the worm's cuticle than other neurochemicals. Alternatively, the delayed onset of paralytic and lethal effects may simply result from the slow actions of ivermectin.

Although the mode of action of ivermectin is not fully understood, many studies have demonstrated that Cl^- channels are involved in its antiparasitic effects [5–9]. In the present study, we used picrotoxin, a Cl^- channel blocker to antagonize ivermectin's effects on locomotor behaviors. Our results were consistent with the involvement of Cl^- channels. However, the antagonism of picrotoxin to ivermectin was effective only in a limited range of concentration (Fig. 3) and time scales. In our preliminary experiments, picrotoxin failed to reverse ivermectin-induced effects on swimming and crawling if the exposure to ivermectin lasted 3 h. A possible explanation is that picrotoxin at high concentrations has some additional effects mediated by action sites other than Cl^- channels. Picrotoxin (5 mM) potentiates contraction while inhibiting voltage-dependent tubular Ca^{2+} current in frog skeletal muscle fibers [31]; at 10 μM, it decreases the intensity of methylation of phospholipids (phosphatidylethanolamine) in rat olfactory cortex [32]. Another possibility is that ivermectin has action sites other than Cl^- channels [33–35], thus, a Cl^- blocker can only reduce but not abolish the effect of ivermectin.

Ivermectin-sensitive Cl^- channels are present in nerve and/or muscle cells in many invertebrates, such as nematodes, insects, crustaceans, and mollusks [36]. Although their physiological role has not been fully determined, they are reported to be involved in generating rhythmic firing of the neurons within the crustacean stomatogastric ganglion [37,38]. Our results showed that ivermectin decreased swimming frequency and crawling frequency, indicating that ivermectin-sensitive Cl^- channels are involved in the neuropathways that control rhythmic swimming and crawling.

It is interesting that the escape reflex behavior controlled by giant interneuron pathways was still intact even after exposure to 300 nM ivermectin. Our electrophysiological studies confirmed that ivermectin had no effects on the conduction velocity of MGF or LGF, suggesting that ivermectin-sensitive Cl^- channels are not crucially involved in the escape reflex functions of giant interneurons. This is not surprising if one takes a close look at the giant interneuron system. The MGF and LGF pathways are derived from the electrically connected large axons of interneurons in each segment. They function as a syncytium, rapidly conducting nerve action potentials without interruption along their length [39]. In such a straightforward system, the main emphasis seems to be speed and reliability; negative feedback via inhibition may be unnecessary or inconsequential. On the other hand, some locomotor behaviors controlled by nongiant interneuron pathways (swimming and crawling) are slower, rhythmic, and probably subject to modulatory influence. Specific networks of neurons in an animal's central nervous system, which control coordinated (and often rhythmic) pattern of movements, are termed central pattern generators [40]. Negative feedback and other modulatory controls are usually utilized in such networks.

In conclusion, our results demonstrated that sublethal behavioral effects were much more sensitive endpoints than was mortality in assessing ivermectin's potential neurobiological and ecological impacts; locomotor behaviors controlled by nongiant interneuron pathways were sensitive to ivermectin, whereas those controlled by giant interneurons did not appear affected at the concentrations studied; and Cl^- channels appeared to be involved in ivermectin's inhibitory effects.

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