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Avermectin-Sensitive Chloride Currents Induced by *Caenorhabditis elegans* RNA in *Xenopus* Oocytes

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SUMMARY

Avermectins are a family of potent broad-spectrum anthelmintic compounds, which bind with high affinity to membranes isolated from the free-living nematode *Caenorhabditis elegans*. Binding of avermectins is thought to modulate chloride channel activity, but the exact mechanism for anthelmintic activity remains to be determined. In this report, the properties of an avermectin-sensitive membrane current were evaluated in *Xenopus laevis* oocytes that were injected with poly(A)$^+$ RNA from *C. elegans*. In such oocytes, avermectins increased inward membrane current at a holding potential of −80 mV. An avermectin analog without anthelmintic activity had no effect. Half-maximal activation of current was observed with 90 nM avermectin. The reversal potential for avermectin-sensitive current was −19.3 ± 1.9 mV, and it shifted with external chloride, as expected for a chloride current. Avermectin increased membrane current in *C. elegans*-injected oocytes that were also injected with the Ca^{2+} chelator ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. The response to avermectin was greatest in the 1.0–2.5-kilobase class of size-fractionated *C. elegans* poly(A)$^+$ RNA. Oocytes that responded to avermectin were insensitive to γ-aminobutyric acid and the avermectin-induced current was blocked by picrotoxin.

The avermectins are a class of macrocyclic lactones, originally isolated from *Streptomyces avermitilis* (1), that are potent anthelmintic and insecticidal compounds with low toxicity in mammals (2–4). The semisynthetic derivative ivermectin is widely used in veterinary medicine as an anthelmintic (for review, see Ref. 5) and in humans to control *Onchocerca volvulus*, the causative agent in river blindness (6, 7).

Although avermectins have been studied in a variety of model systems, the site and mechanism of action remain elusive (for review, see Ref. 8). In the parasitic nematode *Ascaris suum*, avermectins have been shown to block transmission between interneurons and motor neurons (9), to open chloride channels that are insensitive to GABA (10), and to block GABA-mediated increases in chloride permeability (10, 11). In arthropods, avermectins increase the membrane permeability to chloride in both muscle and neuron preparations (12–16). Picrotoxin antagonizes the actions of avermectin, suggesting modulation of GABA-gated chloride channels (12, 14–16). However, avermectins also increase the chloride permeability of tissues without GABA receptors (13, 16) and directly open a channel gated by glutamate and acetylcholine in crayfish stomach muscle (17).

Caenorhabditis elegans are free-living nematodes that are highly sensitive to avermectins (9, 18). Specific high affinity avermectin binding sites have been identified and characterized in this organism (18). Unfortunately, the small size of *C. elegans* has precluded electrophysiologic study. As an alternative, we used *Xenopus laevis* oocytes (19, 20) as a surrogate expression system for *C. elegans* proteins. We demonstrate avermectin-sensitive chloride currents in *Xenopus* oocytes injected with *C. elegans* poly(A)$^+$ RNA.

Materials and Methods

Solutions and drugs. Ivermectin (22,23-dihydroavermectin $B1_a$), $IVMPO_4$, and octahydroavermectin (3,4,8,9,10,11,22,23-octahydroavermectin B_1) were supplied by Dr. H. Mrozik, Merck Sharp and Dohme Research Laboratories (Rahway, NJ) and dissolved as a 1 mM stock solution in DMSO. The maximum bath concentration of DMSO used in this study was 0.1%. DMSO (1%) had no effect on uninjected oocytes or on oocytes injected with *C. elegans* poly(A)$^+$ RNA. Flufenamic acid and picrotoxin were made as 50 mM stock solutions in ethanol. The maximum bath concentration of ethanol (1.0%) had no effect on uninjected oocytes (21) or on oocytes injected with *C. elegans* poly(A)$^+$ RNA. All other drugs and chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO).

The solutions used for isolating and recording from *Xenopus* oocytes are listed in Table 1. Unless otherwise indicated, recordings were made in saline solution.

Oocyte preparation and injection. Oocytes were prepared and injected with methods similar to those described by Leonard *et al.* (22). In brief, adult female *X. laevis* were anesthetized with 0.17% tricaine

ABBREVIATIONS: GABA, γ-aminobutyric acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; DMSO, dimethylsulfoxide; $IVMPO_4$, Ivermectin-4″-*O*-phosphate, monosodium salt, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobases.

TABLE 1
Composition of solutions
Solutions were brought to pH 7.5 with NaOH.

Component	Concentration				
	OR-2	ND-96	Saline	Low Cl^-	Low Na+
			mM		
NaCl	82.5	96	96		19
KCl	2	2	2		2
K_2SO_4				1	
$MgCl_2$	1	1	1	1	1
$CaCl_2$		1.8	1.8	1.8	1.8
HEPES	5	5	5	5	5
N-Methyl-D-glucamine					77
HCl					77
Sodium methanesulfonic acid				96	
Sodium pyruvate		2.5			
Theophylline		0.5			
Gentamicin		0.1			

methanesulfonate, and the ovaries were surgically removed and placed in a dish containing OR-2. Ovarian lobes were broken open, rinsed several times, and gently shaken in OR-2 containing 0.2% collagenase (type 1A; Sigma), for 2–5 hr. When approximately 50% of the follicular layers were removed, stage V and VI oocytes were selected and placed in ND-96 for 24–48 hr before injection. Oocytes were injected with 50–70 nl of poly(A)$^+$ RNA (1 mg/ml) and incubated for 2–3 days in ND-96 before recording. Control oocytes were injected with 50 nl of water. Incubations and collagenase digestion were carried out at 18°. For some experiments, EGTA was injected 1–5 hr before recording (50 nl of a 15 mM solution, pH to 7.5 with KOH.

***C. elegans* RNA isolation.** *C. elegans* cultures were maintained on *Escherichia coli*-seeded agar Petri dishes and isolated by flotation on 60% sucrose, as described by Sulston and Hodgkin (23). To prevent mRNA degradation, *C. elegans* preparations (20 ml) were rapidly frozen in liquid N_2 and ground with a mortar and pestle while submerged in liquid N_2. A solution (50 ml) containing 6 M guanidinium isothiocyanate, 3% sodium sarkosyl, 1.4 M β-mercaptoethanol/H_2O-saturated phenol (1:1) was added to the ground tissue, which was allowed to thaw. The viscous suspension was sheared by two or three passages through an 18-gauge needle. Forty milliliters of a solution containing 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 0.1 M sodium acetate (TES buffer) and 140 ml of chloroform/isoamyl alcohol (24:1) were added, and the solution was heated to 60°, shaken vigorously for 15 min, cooled on ice, and centrifuged for 10 min at 2000 × *g*. The aqueous phase was re-extracted with phenol/chloroform/isoamyl alcohol (25:24:1) at 60°. The aqueous phase was extracted two more times with chloroform/isoamyl alcohol and precipitated with 2.5 volumes of ethanol for 12 hr at −20°. The pellets were resuspended in 10 ml of distilled water, and the mRNA was precipitated with 70 ml of 4 M LiCl at 4° for 12 hr and centrifuged at 11,000 × *g* for 90 min. The pellet was resuspended, precipitated, and washed twice with ethanol (24). All solutions and labware used for the isolation and handling of RNA were treated with diethylpyrocarbonate (24).

Rat and chick brains were homogenized in 5 M guanidine thiocyanate, and the RNA was precipitated with LiCl and purified using methods previously described (25).

C. elegans, rat brain, and chick brain poly(A)$^+$ RNA were isolated by two rounds of purification on an oligo(dT)-cellulose column (Pharmacia) (24). Poly(A)$^+$ RNA was size-fractionated on a 10–30% sucrose gradient with methods similar to those previously described by Sumikawa *et al.* (26). The RNA (150 μg) was centrifuged through the sucrose gradient for 24 hr at 173,000 × *g*, in a SW40 Beckman ultracentrifuge rotor. Aliquots of 0.45 ml were collected from the top of the gradient and precipitated with 0.3 M sodium acetate and 2 volumes of ethanol at −20°.

Northern analysis of *C. elegans* RNA. Aliquots containing 3% of each sucrose gradient fraction were electrophoresed on a 1.5% agarose-formaldehyde gel, blotted to nitrocellulose, and hybridized at 42° in 10 ml of 50% formamide buffer, as described (24). The probe, 5 × 10^6 cpm of ^{32}P-labeled first-strand cDNA, was synthesized from unfractionated *C. elegans* poly(A)$^+$ RNA using a cDNA synthesis kit (Amersham). The blots were washed at 65° in a solution containing 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% sodium dodecyl sulfate, air dried, and exposed to X-ray film.

Electrophysiology. Oocytes were voltage-clamped at room temperature using a standard two-microelectrode amplifier (Dagan 8500, Minneapolis, MN). Pipettes were filled with 3 M KCl and had resistances between 0.5 and 2 mΩ. The bath was connected to a ground Ag/AgCl electrode through a 3 M KCl/agar bridge. Data was acquired and analyzed using pCLAMP with a TL-1 interface (Axon Instruments). For most experiments, membrane current at −80 mV was measured. These data were filtered at 3 kHz, sampled at 16.6 Hz, and digitally filtered at 50 Hz for display. Exceptions are noted in the text and figure legends.

A Plexiglass chamber (volume, 600 μl) was constantly perfused at a rate of 10 ml/min. The solution changer had a dead time of about 5 sec, and drugs were usually applied for 1 min. The amplitude of drug-sensitive current was determined by subtracting the holding current at −80 mV from the peak current obtained in the presence of drug.

Current-voltage relationships were determined over the voltage range of −110 to +80 mV. Either 3-sec voltage ramps or 40-msec voltage steps were used. The filter frequency was 3 kHz, and the sampling rates were 160 Hz and 10 kHz for ramps and voltage steps, respectively. With both protocols, currents in drug-free solutions were subtracted from currents in the presence of drug, to obtain drug-sensitive current.

The reversal potential of the endogenous calcium-activated chloride current [$I_{Cl(Ca)}$] was determined using tail current analysis, as described previously (27).

Data analysis and curve fitting. The concentration-effect curves were fit by applying a nonlinear least square fitting program (28) to a modified Michaelis-Menten equation:

$$\% \text{ of maximal effect} = 1/[1 + (K_d/[D])^n] \quad (1)$$

where $[D]$ is the drug concentration, K_d is the drug concentration for half-maximal effect, and n is the Hill coefficient. Free parameters were K_d and n.

Current-voltage relationships were fit (28) to the Goldman-Hodgkin-Katz equation (29, 30), with the assumptions that the membrane was selectively permeable to chloride and that $[Cl^-]_i$ was 40 mM (19).

$$I = P_{Cl} F \Psi \frac{[Cl^-]_o - [Cl^-]_i e^{\Psi}}{1 - e^{\Psi}} \quad (2)$$

with

$$\Psi = -VF/RT$$

where P_{Cl} is the membrane permeability to Cl^- and V is the membrane potential. R, F, and T have their usual meanings. The free parameter was P_{Cl}.

All averaged data that are included in text and figures are mean ± standard error. Paired or unpaired t tests were used for determination of statistical significance. When possible, statistical comparisons were made from oocytes taken from the same donor.

Results

Ivermectin induces an inward membrane current in oocytes injected with *C. elegans* poly(A)$^+$ RNA. Ivermectin (1 μM) evoked inward membrane currents of −229 ± 39 nA (n = 10) in *Xenopus* oocytes injected with *C. elegans* poly(A)$^+$ RNA (Fig. 1A). The effect was essentially irreversible, with only 10% of the current recovering when the bath was washed for up to 40 min (n = 3). The ivermectin-induced current had

a slow onset, with peak responses occurring at 49 ± 4 sec (n = 9). There was no response to ivermectin in water-injected oocytes (n = 20) (Fig. 1A), uninjected oocytes (n = 10), or oocytes injected with poly(A)$^+$ RNA from rat brain (n = 10) or chick brain (n = 3).

The bath concentrations of avermectins are difficult to accurately determine because of the hydrophobic properties of the drugs (9, 31); consequently, a water-soluble avermectin derivative, $IVMPO_4$, was used. Similar to ivermectin, $IVMPO_4$ (1 μM) slowly and irreversibly activated inward currents in *C. elegans*-injected oocytes (−241 ± 50 nA; n = 20) but not in oocytes injected with water (n = 20) or chick brain (n = 4) or rat brain (n = 5) poly(A)$^+$ RNA (Fig. 1B).

$IVMPO_4$ activated current in a concentration-dependent manner (Fig. 2). The concentration-response curve was determined by recording responses to a submaximal (test) and maximal (3 μM) concentration of $IVMPO_4$ in the same oocyte. The response recorded at the test concentration was normalized to the response induced by 3 μM $IVMPO_4$. The data show a dependence on concentration, with a K_d of 90 nM and a Hill coefficient of 1.1.

Octahydroavermectin, a biologically inactive analog with very poor receptor binding (18), had no effect on oocytes injected with *C. elegans* poly(A)$^+$ RNA (n = 4) (Fig. 3). These oocytes subsequently responded (−91 ± 30 nA) to 200 nM $IVMPO_4$ (Fig. 3).

To assess the role of endogenous oocyte transcription on the expression of ivermectin-sensitive current, we preincubated oocytes with 50 μg/ml actinomycin D for 3 hr before injecting *C. elegans* poly(A)$^+$ RNA. Oocytes were subsequently incubated in the presence of actinomycin D for 48 hr before recording (25). At 48 hr, incorporation of [^{3}H]uridine into oocyte RNA was reduced 55% in the actinomycin-treated group (data not shown). Currents activated by 500 nM $IVMPO_4$ were the same in the control (−315 ± 69 nA; n = 11) and actinomycin D-treated groups (−312 ± 51 nA; n = 10).

Ivermectin increases the oocyte membrane permeability to chloride. Oocytes injected with *C. elegans* poly(A)$^+$ RNA had a resting potential of −38 mV. After application of $IVMPO_4$, the resting potential shifted to −24 mV (Table 2). This value is close to the Nernst potential (E_{Cl}) calculated for a chloride electrode (−24.1 mV, 40 mM $[Cl^-]_i$). In uninjected and water-injected oocytes, the resting potential was approximately −43 mV before and after application of $IVMPO_4$ (Table 2).

Fig. 4 shows an experiment in which the current-voltage relationship for $IVMPO_4$-sensitive current was measured in saline and in low Cl^- (6 mM $[Cl^-]_o$) solutions. Fig. 4A shows representative records of membrane current elicited during 40-msec voltage steps in saline. $IVMPO_4$ increased time-independent membrane current during the voltage step and at the holding potential. $IVMPO_4$-sensitive current was time independent over the voltage range of −110 to +80 mV, even when 3-sec voltage steps were used.

In saline the relationship for $IVMPO_4$-sensitive current outwardly rectified and reversed at −24 mV (Fig. 4B). In low Cl^-

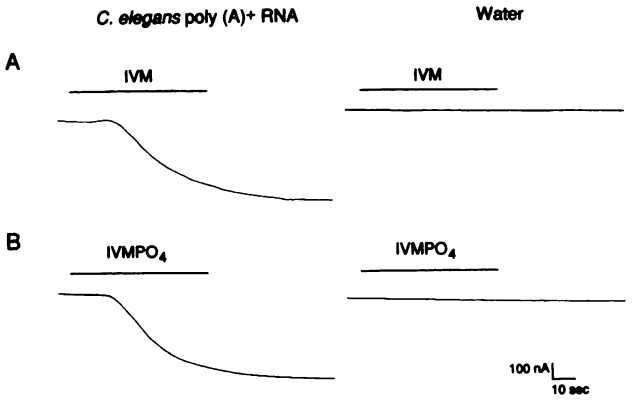

Fig. 1. Avermectins activate an inward membrane current. Membrane currents from oocytes injected with *C. elegans* poly(A)$^+$ RNA (*left*) and with water (*right*). *Solid lines above current traces*, the time of drug application. A, Perfusion of 1 μM ivermectin (*IVM*). B, Perfusion of 1 μM $IVMPO_4$.

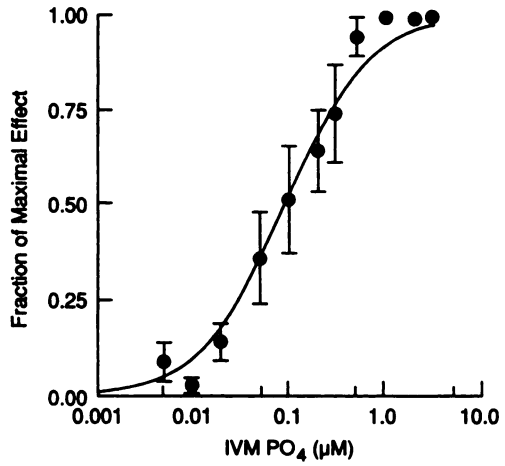

Fig. 2. Concentration-response curve for $IVMPO_4$. Membrane currents were measured in *C. elegans* poly(A)$^+$ RNA-injected oocytes. For each experiment, responses from submaximal (test) and maximal (3 μM) concentrations of $IVMPO_4$ were recorded. Fraction of maximal effect was determined by normalizing the response at the test concentration to the response at 3 μM. The curve represents a fit to Eq. 1. Data without error bar indicate that the standard error was less than the symbol size. Three to nine experiments were done for each concentration.

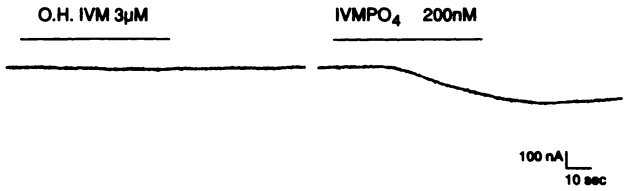

Fig. 3. An inactive analog of avermectin fails to activate current. Membrane currents were measured in an oocyte injected with *C. elegans* poly(A)$^+$ RNA. *O.H. IVM*, octahydroavermectin. The time between recordings was 2 min.

TABLE 2

Membrane potential before and after $IVMPO_4$

Oocytes[a]	Membrane potential
	mV
Water-injected	
Untreated	−42.0 ± 2.0
$IVMPO_4$, 500 nM	−44.0 ± 2.0
C. elegans poly(A)$^+$ RNA-injected	
Untreated	−38.0 ± 2.0[b]
$IVMPO_4$, 500 nM	−24.0 ± 1.0[c]

[a] n = 25 for all groups.
[b] Not different from water-injected untreated group (unpaired t test).
[c] Significantly less than *C. elegans*-injected untreated group ($p < 0.0001$; paired t test).

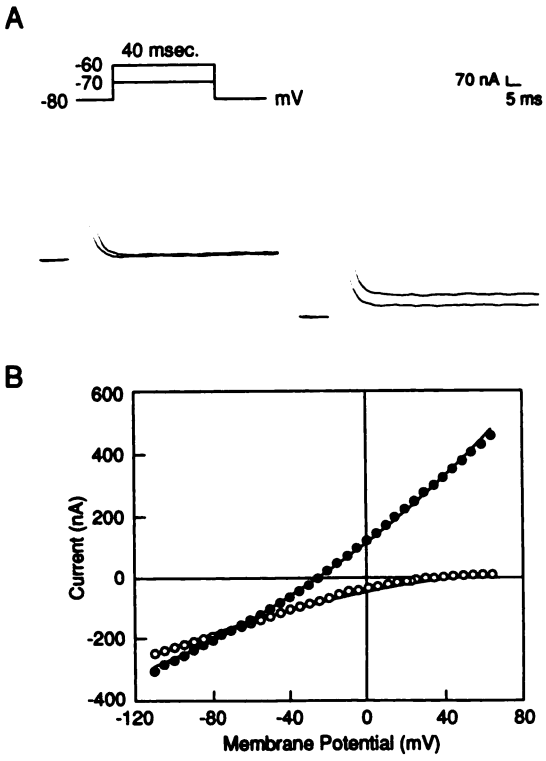

Fig. 4. Current-voltage relationship for $IVMPO_4$-sensitive current. Membrane currents were measured in an oocyte injected with *C. elegans* poly(A)$^+$ RNA. A, Voltage protocol and membrane current traces in the absence (*left*) and presence (*right*) of 500 nM $IVMPO_4$ (104 mM $[Cl^-]_o$). B, Current-voltage relationships for $IVMPO_4$-sensitive current in 104 mM (●) and 6 mM (○) $[Cl^-]_o$. $IVMPO_4$-sensitive current was determined by subtracting data in drug-free solutions from data in the presence of $IVMPO_4$ (data at 10–15 msec used for analysis). *Smooth curves*, fits to the constant field equation (Eq. 2), with P_{Cl} of 1.8×10^{-11} cm^3/sec (104 mM $[Cl^-]_o$) and 1.5×10^{-11} cm^3/sec (6 mM $[Cl^-]_o$).

the relationship rectified inwardly and reversed at +45 mV. This shift in curvature and reversal potential with $[Cl^-]_o$ was consistent with the predictions of the constant field equation (smooth curves in Fig. 4B) for a membrane selectively permeable to chloride (29, 30). When that bathing solution was changed from saline to low Na^+ (19 mM $[Na^+]_o$), neither the shape of the current-voltage relationship nor the reversal potential was altered.

The reversal potential for $IVMPO_4$-sensitive current was -19.3 ± 1.9 mV ($n = 18$). In low Cl^- solution, the reversal potential shifted to 43.4 ± 5.2 mV ($n = 5$). In saline, the reversal potential for $IVMPO_4$-sensitive current was slightly less than the reversal potential of an endogenous chloride channel [$I_{Cl(Ca)}$] measured under the same conditions in the same batch of oocytes (-23.8 ± 1.9 mV; $n = 17$) ($p < 0.05$).

Flufenamic acid is a nonsteroidal anti-inflammatory compound that blocks a variety of chloride channels, including the mammalian $GABA_A$ receptor, $I_{Cl(Ca)}$ in *Xenopus* oocytes, and the voltage-dependent chloride channel from *Torpedo* electroplax (21, 32). Flufenamic acid (100 μM) partially ($62 \pm 12\%$; $n = 4$) and reversibly inhibited $IVMPO_4$-sensitive current (Fig. 5). Flufenamic acid had no effect on current at −80 mV in the absence of $IVMPO_4$.

$IVMPO_4$ activated a similar current in *C. elegans* poly(A)$^+$ RNA oocytes that were also injected with EGTA to buffer intracellular calcium. Buffering of intracellular calcium was confirmed by showing blockade of the endogenous calcium-activated chloride current [$I_{Cl(Ca)}$] (Fig. 6). Fig. 6A shows records of membrane current evoked with 3-sec voltage steps to −10 and +10 mV. Time-dependent $I_{Cl(Ca)}$ was evoked in response to the entry of calcium through voltage-gated calcium channels (19, 27). Time-dependent current was not activated in the *C. elegans* poly(A)$^+$ RNA-injected oocyte that was also injected with EGTA (Fig. 6A). In non-EGTA-injected oocytes the amplitude of $I_{Cl(Ca)}$ was 350 ± 50 nA at +10 mV ($n = 5$). In EGTA-injected oocytes there was no time dependence typical of $I_{Cl(Ca)}$, and the amplitude was 43 ± 22 nA ($n = 5$).

In EGTA-injected oocytes, application of 500 nM $IVMPO_4$ activated an inward membrane current at −80 mV (Fig. 6B). The mean amplitude, reversal potential, and current-voltage relationship for this $IVMPO_4$-sensitive current were the same as for those in oocytes not injected with EGTA ($n = 4$).

Ivermectin-sensitive current is not activated by GABA. GABA (10 μM to 1 mM) and glycine (0.1–1 mM) failed to activate currents in *C. elegans* poly(A)$^+$ RNA-injected oocytes that showed responses to $IVMPO_4$ (Fig. 7A). GABA was also ineffective when applied during and/or after activation of current with $IVMPO_4$.

Picrotoxin (500 μM) inhibited $IVMPO_4$ activated current $81 \pm 7\%$ ($n = 5$, Fig. 7B). Half maximal block was observed with approximately 100 μM picrotoxin. $IVMPO_4$ activated current was insensitive to bicuculline (10–100 μM), the $GABA_A$ receptor agonist muscimol (100 μM, and the benzodiazepine derivative flunitrazepam (10 μM).

Sucrose density fractionation of *C. elegans* poly(A)$^+$ RNA. *C. elegans* poly(A)$^+$ RNA was size-fractionated into 40 fractions by sucrose gradient centrifugation, and the fractions were tested by injection into *Xenopus* oocytes (Fig. 8). Oocytes injected with approximately 13 ng of RNA from fractions 14–18 elicited $IVMPO_4$-sensitive currents (Fig. 8A). No response to $IVMPO_4$ was observed in oocytes injected with the remaining fractions from the gradient. The RNA in fraction 15 induced the greatest response to $IVMPO_4$. The time course, voltage dependence, and reversal potential were similar to those described for unfractionated poly(A)$^+$ RNA. The molecular weight of the RNA was determined to be 1.0–2.5 kb by electrophoresis on a denaturing agarose gel (Fig. 8B).

Discussion

Avermectin-induced increases in chloride permeability result from expression of relevant protein(s) from *C. elegans* poly(A)$^+$ RNA and not from translation, activation, and/or modulation of endogenous oocyte proteins. Several lines of evidence support this conclusion. Firstly, avermectin does not activate currents in uninjected or water-injected oocytes. Secondly, the response to avermectin is unchanged if activation of endogenous chloride channels is blocked with intracellular EGTA. This experiment also serves to rule out expression of an avermectin receptor that couples through GTP-binding proteins to elevate intracellular calcium (19, 20). Thirdly, expression of *C. elegans* RNA does not lead to transcription of endogenous oocyte genes, because avermectin activates currents in oocytes where endogenous transcription was reduced by 55% with actinomycin D. Finally, the characteristics of the oocyte-expressed avermectin-sensitive current are similar to those described for the membrane-associated *C. elegans* avermectin binding site (18). Binding data show that avermectin forms an essentially irreversible

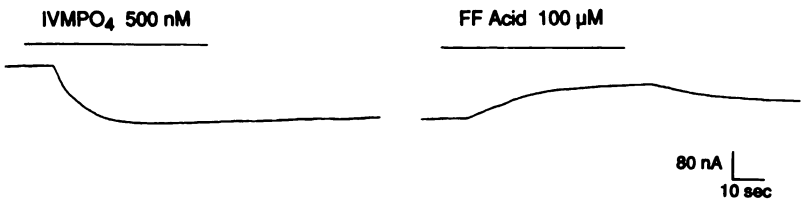

Fig. 5. Ivermectin-sensitive current is blocked by flufenamic acid. Membrane currents were measured in an oocyte injected with *C. elegans* poly(A)$^+$ RNA. *FF Acid*, flufenamic acid. The time between recordings was 2 min.

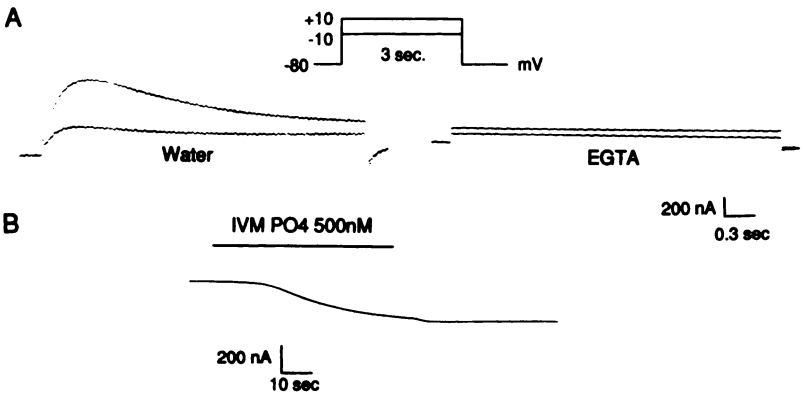

Fig. 6. IVMPO$_4$ does not activate $I_{Cl(Ca)}$. A, Voltage protocol and membrane current records from an oocyte injected with water (*Water*) and one injected with *C. elegans* poly(A)$^+$ RNA, then EGTA 1 hr before recording (*EGTA*). Data were sampled at 160 Hz and filtered at 3 kHz. B, Membrane current at a holding potential of −80 mV from the EGTA-injected oocyte shown in A.

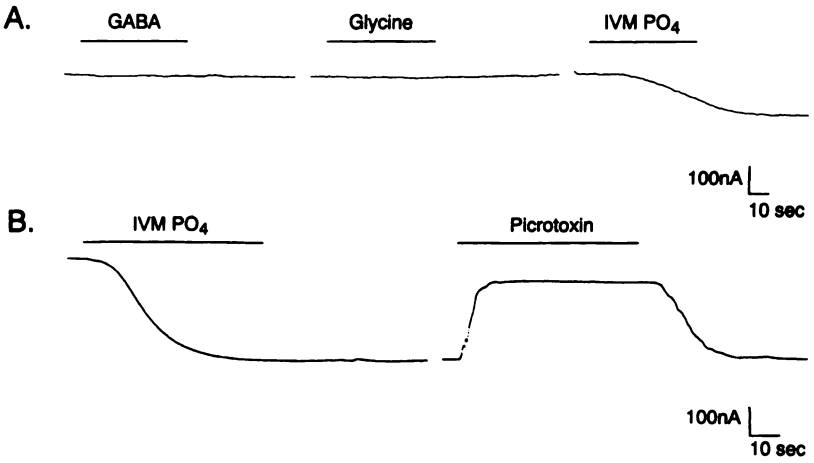

Fig. 7. IVMPO$_4$-induced current is not sensitive to GABA and is blocked with picrotoxin. Membrane currents from *C. elegans* poly(A)$^+$ RNA-injected oocytes. IVMPO$_4$ (500 nM). The time between recordings was 1 min. A, GABA (1 mM) and Glycine (1 mM). B, Picrotoxin (500 µM).

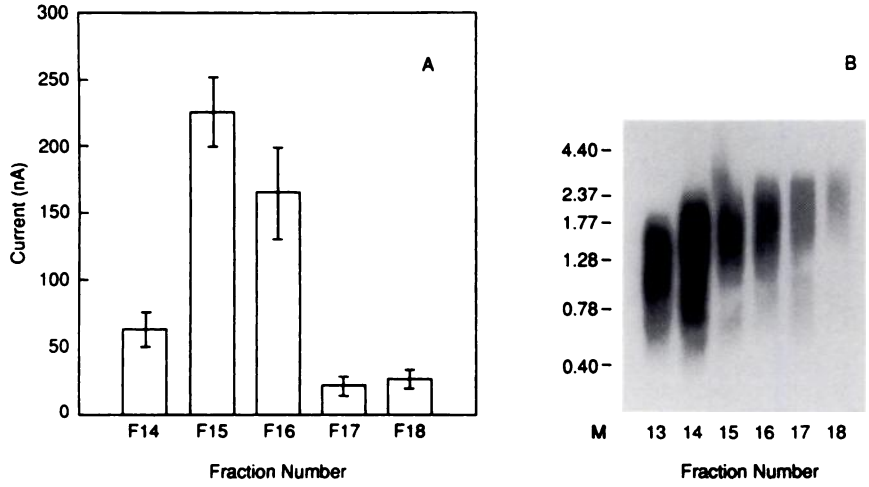

Fig. 8. Sucrose gradient fractionation of mRNA. A, Oocytes were injected with approximately 13 ng of fractionated RNA and perfused with 500 nM IVMPO$_4$. The mean ± standard error of membrane currents are shown. B, Fractionated RNA was electrophoresed on a 1.5% agarose-formaldehyde gel and hybridized to ^{32}P-labeled cDNA (see Materials and Methods). The molecular weight markers (*M*) are noted in kb.

complex with its receptor (18), and we find that activation of chloride current by avermectin is essentially irreversible. Octahydroavermectin does not bind to the *C. elegans* avermectin receptor (18) and does not activate currents in our system. In addition, Schaeffer and Haines (18) found no effect of GABA or bicuculline on avermectin binding and no evidence for benzodiazepine binding in *C. elegans* membranes. Similarly, we find no effects of these agents, whether applied before, during, or after activation of avermectin-sensitive current.

The resting potential of oocytes after application of aver-

mectin and the reversal potential for avermectin-sensitive current were close to V_{Cl}. These data, taken together with the dependence of reversal potential on external chloride, strongly suggest expression of a chloride channel. The avermectin-sensitive current was time independent and showed a mild voltage dependence that rectified with external chloride, as predicted from constant field theory (29, 30). Using voltage steps from −150 to +150 mV, we found no evidence for activation of this current in the absence of avermectin. These data suggest that avermectins do not modulate a voltage-gated channel and that they probably activate a background and/or ligand-gated channel.

The reversal potential for avermectin-sensitive current was slightly less negative than that of the endogenous chloride channel [$I_{Cl(Ca)}$], as well as the V_{Cl}. Therefore, the avermectin-sensitive "channel" may be less selective for chloride than is $I_{Cl(Ca)}$. It does not appear that sodium permeates the channel, because reducing $[Na^+]_o$ by 80% had no effect on the reversal potential.

Activation of current by avermectin was relatively slow, compared with activation of neurotransmitter-gated channels. In our system, peak responses to GABA occur at 6.0 ± 1.0 sec (n = 11) when oocytes are injected with rat brain poly(A)$^+$ RNA. Slow onset for the actions of avermectin have been reported (10, 13) and may result from limited diffusion to a site within the membrane (33). However, slow onsets are also observed with ligands that couple through second-messenger pathways (19, 20). Our data do not distinguish between these two possibilities. However, it does not appear that avermectin acts through a pertussis toxin-sensitive GTP-binding protein, because incubation of oocytes for 24 hr with 2 μg/ml pertussis toxin did not alter the response to avermectin (data not shown).

The mechanism of action of avermectins in nematodes is unclear (8). Effects reported include block (10, 11) and enhancement (8, 9) of GABA-mediated chloride current and opening of GABA-insensitive chloride channels (10). Our data strongly support a mechanism whereby avermectin directly opens a chloride channel that is insensitive to GABA. We find no evidence for activation or modulation of avermectin-sensitive current with GABA or other GABA receptor ligands. We also find no expression of GABA-sensitive membrane current over the 72-hr incubation time used in this study. However, the vertebrate $GABA_A$ receptor is multisubunit (for review, see Ref. 34), and it is plausible that the subunit for the *C. elegans* GABA binding site is not translated in oocytes, whereas the avermectin-sensitive subunits are expressed.

In *Xenopus* oocytes expressing chick brain $GABA_A$ receptors, avermectins have no effect when applied alone (35). In contrast, when applied with GABA avermectins increase the response to submaximal GABA concentrations, apparently by decreasing the K_d for GABA (35). This modulatory effect of avermectin on mammalian $GABA_A$ receptors is clearly different from the direct activation of a GABA-insensitive current observed with nematode RNA in this study.

Our results are consistent with previous reports, which show that avermectins directly increase the membrane permeability to chloride (13–17). These studies show that avermectins are effective when applied alone and do not require addition of exogenous transmitters. Also, several studies show that picrotoxin reverses the effects of avermectins (12, 15, 16, 17) in arthropods as well as in *Ascaris* (9). It is interesting to note that the *Ascaris* GABA receptor is insensitive to picrotoxin (11).

Reports in arthropods postulate that avermectins act at a site that is distinct from the GABA receptor (13, 14, 16, 17). For example, Zufall *et al.* (17) have shown that avermectin activates a picrotoxin-sensitive multitransmitter (glutamate, quisqualate, ibotenate, and nicotinic agonist)-gated chloride channel in crayfish stomach muscle. It will be important to determine what transmitter, if any, gates the *C. elegans* avermectin receptor.

The poly(A)$^+$ RNA that encodes the proteins for $IVMPO_4$-sensitive current resides in a fraction of RNA that ranges in size from 1.0 to 2.5 kb. This is a large pool of different size classes of RNA and, therefore, we cannot rule out the possibility that the avermectin-sensitive protein may be encoded by multiple RNA species. For example, the mammalian $GABA_A$, kainate, and nicotinic acetylcholine receptors consist of multiple subunits (34, 36). Further fractionation of RNA or isolation of individual cDNA clones will be required to determine the composition of the *C. elegans* protein.

We demonstrate that translation of a 1.0–2.5-kb size class of *C. elegans* poly(A)$^+$ RNA renders *Xenopus* oocytes responsive to avermectins. The data strongly support the expression of an ion channel that is highly permeable to chloride. *Xenopus* oocytes have been useful in the examination of the properties of chloride channels gated by GABA and glycine (34) and in the cloning of a voltage-dependent chloride channel from *Torpedo marmorata* (37). The current expressed in the present study does not have the voltage- and time-dependent properties of the *Torpedo* channel (37), and it is not activated by GABA or glycine. Therefore, the avermectin-sensitive current may be due to expression of a novel invertebrate chloride channel. The *Xenopus* oocyte expression system may prove very useful to clone and characterize the properties of this and other nematode ion channels that, until now, were not accessible for electrophysiologic evaluation.

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References

1. Burg, R. W., B. M. Miller, E. E. Baker, J. Birnbaum, J. A. Currie, R. Harman, V. L. Kong, R. L. Monaghan, G. Olson, I. Putter, J. D. Tunac, H. Wallick, E. O. Stapley, R. Oiwa, and S. Omura. Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. *Antimicrob. Agents Chemother.* **15**:361–367 (1979).
2. Egerton, J. R., D. A. Ostlind, L. S. Blair, D. H. Eary, D. Suhayda, S. Cifelli, R. F. Riek, and W. C. Campbell. Avermectins, a new family of potent anthelmintic agents: efficacy of the B1a compounds. *Antimicrob. Agents Chemother.* **15**:372–378 (1979).
3. Ostlind, D.A., S. Cifelli, and R. Lang. Insecticidal activity of the antiparasitic avermectins. *Vet. Rec.* **105**:168 (1979).
4. Campbell, W. C., and G. W. Benz. Ivermectin: a review of efficacy and safety. *J. Vet. Pharmacol. Ther.* **7**:1–16 (1984).
5. Campbell, W. C., ed. *Ivermectin and Abamectin.* Springer-Verlag, New York (1989).
6. Greene, B. M., K. R. Brown, and H. R. Taylor. Use of ivermectin in humans, in *Ivermectin and Abamectin* (W. C. Campbell, ed.). Springer-Verlag, New York, 311–323 (1989).
7. Taylor, H. R., M. Pacque, B. Munoz, and B. M. Greene. Impact of mass treatment of *Oncocerciasis* with ivermectin on the transmission of infection. *Science (Washington D. C.)* **250**:116–118 (1990).
8. Turner, M. J., and J. M. Schaeffer. Mode of action of ivermectin, in *Ivermectin and Abamectin* (W. C. Campbell, ed.). Springer-Verlag, New York, 73–88 (1989).
9. Kass, I. S., C. C. Wang, J. P. Walrond, and A. O. W. Stretton. Avermectin B_{1a}, a paralyzing anthelmintic that affects interneurons and inhibitory motor neurons in *Ascaris*. *Proc. Natl. Acad. Sci. USA* **77**:6211–6215 (1980).

10. Martin, R. J., and A. J. Pennington. A patch-clamp study of effects of dihydroavermectin on *Ascaris* muscle. *Br. J. Pharmacol.* **98**:747–756 (1989).
11. Holden-Dye, L. and R. J. Walker. Avermectin and avermectin-derivatives are antagonists at the 4-aminobutyric acid (GABA) receptor on the somatic muscle cells of *Ascaris*: is this the site of anthelmintic action? *Parasitology* **101**:265–271 (1990).
12. Fritz, L. C., C. C. Wang, and A. Gorio. Avermectin B_{1a} irreversibly blocks presynaptic potentials at the lobster neuromuscular junction by reducing muscle membrane resistance. *Proc. Natl. Acad. Sci. USA* **76**:2062–2066 (1979).
13. Duce, I. R., and R. H. Scott. Actions of dihydroavermectin B_{1a} on insect muscle. *Br. J. Pharmacol.* **98**:747–756 (1989).
14. Tanaka, K., and F. Matsumura. Action of avermectin B_{1a} on the leg muscles and nervous system of the American cockroach. *Pestic. Biochem. Physiol.* **24**:124–135 (1985).
15. Mellin, T. N., R. D. Busch, and C. C. Wang. Postsynaptic inhibition of invertebrate neuromuscular transmission by avermectin B_{1a}. *Neuropharmacology* **22**:89–96 (1983).
16. Lees, G., and D. J. Beadle. Dihydroavermectin B1: actions on cultured neurones from the insect central nervous system. *Brain Res.* **366**:369–372 (1986).
17. Zufall, F., C. Franke, and H. Hatt. The insecticide avermectin B_{1a} activates a chloride channel in crayfish muscle membrane. *J. Exp. Biol.* **142**:191–205 (1989).
18. Schaeffer, J. M., and H. W. Haines. Avermectin binding in *Caenorhabditis elegans*: a two-state model for the avermectin binding site. *Biochem. Pharmacol.* **38**:2329–2338 (1989).
19. Dascal, N. Use of the *Xenopus* oocyte system to study ion channels. *Crit. Rev. Biochem.* **22**:317–387 (1987).
20. Lester, H. A. Heterologous expression of excitability proteins: route to more specific drugs? *Science (Washington D.C.)* **241**:1057–1063 (1988).
21. White, M. M., and A. Aylwin. Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. *Mol. Pharmacol.* **37**:720–724 (1990).
22. Leonard, J. P., J. Nargeot, T. P. Snutch, N. Davidson, and H. A. Lester. Ca channels induced in *Xenopus* oocytes by rat brain mRNA. *J. Neurosci.* **7**:875–881 (1987).
23. Sulston, J., and J. Hodgkin. Methods: bulk growth on bacteria, in *The Nematode Caenorhabditis elegans* (W. B. Wood, ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 602–603 (1988).
24. Maniatis, T., E. f. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
25. Sigel, E. Properties of single sodium channels translated by *Xenopus* oocytes after injection with messenger ribonucleic acid. *J. Physiol. (Lond.)* **386**:73–90 (1987).
26. Sumikawa, K., M. Houghton, J. C. Smith, L. Bell, B. M. Richards, and E. A. Barnard. The molecular cloning and characterization of cDNA coding for the α subunit of the acetylcholine receptor. *Nucleic Acids Res.* **10**:5809–5822 (1982).
27. Barish, M. E. A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol (Lond.)* **342**:309–325 (1983).
28. Norby, J., S. Rubenstein, T. Tuerke, C. Farmer, and J. Bennington. Sigmaplot. Jandel Scientific, Corde Madera (1988).
29. Goldman, D. E. Potential, impedance, and rectification in membranes. *J. Gen. Physiol.* **27**:37–60 (1943).
30. Hodgkin, A. L., and B. Katz. The effect of sodium on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.)* **108**:37–77 (1949).
31. Fisher, M. H., and H. Mrozik. Chemistry, in *Ivermectin and Abamectin* (W. C. Campbell, ed.). Springer-Verlag, New York, 1–23 (1989).
32. Miller, C., and M. M. White. A voltage-gated Cl^- conductance channel from *Torpedo electroplax* membrane. *Ann. N. Y. Acad. Sci.* **341**:534–551 (1980).
33. Martin, R. J., J. R. Kusel, and A. J. Pennington. Surface properties of membrane vesicles prepared from muscle cells of *Ascaris suum*. *J. Parasitol.* **76**:340–348 (1990).
34. Dingledine, R., S. J. Myers, and R. A. Nicholas. Molecular biology of mammalian amino acid receptors. *FASEB J.* **4**:2636–2645 (1990).
35. Sigel, E., and R. Baur. Effect of avermectin B_{1a} on chick neuronal γ-aminobutyrate receptor channels expressed in *Xenopus* oocytes. *Mol. Pharmacol.* **32**:749–752 (1987).
36. Nakanishi, N., N. A. Shneider, and R. Axel. A family of glutamate receptor genes: evidence for the formation of heteromultimeric receptors with distinct channel properties. *Neuron* **5**:569–581 (1990).
37. Jentsch, T. J., K. Steinmeyer, and G. Schwarz. Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes. *Nature (Lond.)* **348**:510–490 (1990).

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