0026-895X/91/020326-07$03.00/0

MOLECULAR PHARMACOLOGY, 40:326–332

Solubilization and Characterization of a High Affinity Ivermectin Binding Site from *Caenorhabditis elegans*

D. F. CULLY and P. S. PARESS

Merck & Co., Department of Biochemical Parasitology, Rahway, New Jersey 07065

Received January 18, 1991; Accepted May 13, 1991

SUMMARY

Ivermectin is a member of the avermectin family of compounds that are used to treat helminth and arthropod diseases in humans, domestic animals, and plants. A membrane-bound high affinity ivermectin binding site was extracted from *Caenorhabditis elegans* with the nonionic detergent 1-*O*-*n*-octyl-β-D-glucopyranoside. The free-living nematode *C. elegans* is highly sensitive to the avermectins and was used as a model of parasitic nematodes. The membrane-bound and detergent-solubilized ivermectin binding sites are stable and exhibit high affinity binding, with dissociation constants of 0.11 nM and 0.20 nM, respectively. The maximum binding of [^{3}H]ivermectin is 0.54 pmol/mg of membrane protein and 0.66 pmol/mg of detergent-soluble protein. Kinetic analysis of ivermectin binding shows that the ivermectin binding sites form a slowly reversible complex with ivermectin. The rates of dissociation of [^{3}H]ivermectin with the solubilized and membrane-bound binding sites are 0.005 min^{-1} and 0.006 min^{-1}, respectively. The association rate of the soluble binding site is 0.053 nM^{-1} min^{-1}, slightly slower than that observed for the membrane-bound site, 0.074 nM^{-1} min^{-1}. To characterize the ivermectin binding site, competition experiments were performed by inhibiting [^{3}H]ivermectin binding with several avermectin derivatives and the neurotransmitter γ-aminobutyric acid (GABA). The order of potency was 22,23-dihydroavermectin B_{1a} monosaccharide > 22,23-dihydroavermectin B_{1a} aglycone > 3,4,8,9,10,11,22,23-octahydro B_1 avermectin for both the membrane-bound and NOG-soluble binding sites. GABA did not compete with ivermectin binding, although it has been suggested that ivermectin acts at the GABA-gated chloride channel in some invertebrate systems. Optimum ivermectin binding and assay conditions have been determined. The detergent-soluble ivermectin binding site appears to be negatively charged and has a pI of 4.0 and an apparent M_r in Triton X-100 micelles of 340,000. Detergent solubilization of a high affinity ivermectin binding site will enable the subsequent purification and characterization of a putative site of ivermectin action.

The AVMs are naturally occurring macrocyclic lactones that have been shown to be potent anthelmintic agents (1). The compounds have a broad range of activities and are currently used to treat both nematode and arthropod parasitisms of domestic animals, humans, and plants (for review, see Ref. 2).

The mode of action of IVM, an AVM derivative (3), has been studied extensively in various organisms such as nematodes, crustaceans, and insects (for review, see Ref. 4). However, it has been difficult to determine a mechanism of IVM action from these studies, because several different model systems have been examined, using large variations in IVM concentrations. The current belief is that IVM causes paralysis of the target organism by increasing permeability of membranes to chloride ions. At low IVM concentrations (picomolar to nanomolar), an increase in chloride conductance of both GABA- and non-GABA-innervated muscles has been observed in insect systems (5, 6). The opening of the IVM-sensitive chloride channel is blocked by the ligand-gated chloride channel-blocker picrotoxin in cockroach muscle (6), primary cockroach brain cultures (7), and crayfish muscle (8). Crayfish muscle chloride channels that are activated by IVM and blocked by picrotoxin exhibit the same conductance and kinetic proterties as those activated by glutamate and carbacol (8). Irreversible conductance increases by IVM in locust muscle may be caused by actions on the glutamate H receptor/chloride ion channel (9). Electrophysiology experiments in the parasitic nematode *Ascaris suum* (10, 11) or in crustacean muscle (12, 13) suggest that micromolar amounts of IVM can change the membrane chloride permeability by acting as a GABA antagonist or agonist or by stimulating GABA secretion. Finally, IVM is a potent inhibitor of [^{3}H]muscimol binding in honey bee brain (14). These studies point to IVM acting at a chloride channel that in invertebrates may be gated by GABA, glutamate, or actylcholine. A clearer understanding of the mechanism of action of such an important pharmacological agent is needed.

The soil nematode *Caenorhabditis elegans* is extremely sen-

ABBREVIATIONS: AVM, avermectin; NOG, 1-*O*-*n*-octyl-β-D-glucopyranoside; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PEI, polyethyleneimine; PMSF, phenylmethylsulfonyl fluoride; TX-100, Triton X-100; IVM, ivermectin; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FPLC, fast protein liquid chromatography.

sitive to IVM (15), and large quantities of this organism can readily be grown in the laboratory. A high affinity, saturable, IVM binding site in *C. elegans* has been identified (15). A direct correlation between the *in vivo* potency of AVM derivatives and the affinity of binding in *C. elegans* membrane preparations has been demonstrated (15). In this study, we report the solubilization of this membrane-bound IVM binding site with the nonionic detergent NOG. Detergent extraction did not alter the affinity of binding to [^{3}H]IVM or the concentration of binding sites. The kinetics of association and dissociation of IVM and this high affinity binding site are similar for the membrane-bound form and the soluble form. In addition, several analogs of AVM, known to have different efficacies *in vivo*, exhibit similar binding characteristics with the membrane-bound and soluble forms of the binding site. We conclude that a high affinity IVM binding site of *C. elegans* has been detergent solubilized. In addition, we have examined the biochemical characteristics of the IVM binding site and in the process have determined the charge and estimated size of the complex.

Experimental Procedures

Materials. IVM is composed of at least 80% 22,23-dihydroavermectin B_{1a} and < 20% 22,23-dihydroavermectin B_{1b}. For this study, we used only the pure B_{1a} constituent of IVM. The AVMs 22,23-dihydroavermectin B_{1a} aglycone and 22,23-dihydroavermectin B_1 monosaccharide were synthesized and their composition was verified on analytical thin layer chromatography, as described (3). These AVMs and 3,4,8,9,10,11,22,23-octahydroavermectin B_1 were obtained from Dr. H. Mrozik, Merck, Sharp & Dohme Research Laboratories (Rahway, NJ). [^{3}H]22,23-Dihydroavermectin B_{1a} ([^{3}H]IVM) was labeled at the 22,23-position by catalytic hydrogenation with tritium gas, to a specific activity of 51.9 Ci/mmol. [^{3}H]IVM was > 95% pure, as determined by thin layer chromatography on silica gel 60-F 254 (E. M. Laboratories, Inc.). GABA was obtained from Sigma. The detergents NOG and TX-100 were of high purity (Pierce). Azolectin (soybean phospholipid) was obtained from Associated Concentrated (Woodside, NY). All other reagents were of the highest purity commercially obtainable.

Nematode cultures. The wild-type Bristol strain of *C. elegans* (N2), originally described by Brenner (16), was used. Worms were grown either on agar plates or in liquid cultures with *Escherichia coli* as a food source and were harvested as previously described (17). Large preparations of liquid cultures (150 liters) were prepared in air-lift fermentation flasks. The *C. elegans* preparations were cleaned from excess *E. coli* protein by flotation in 35% sucrose and were washed in 0.1 M NaCl (17).

***C. elegans* tissue preparation.** For small preparations, approximately 5×10^5 (1 ml) worms were resuspended in 2 ml of 50 mM HEPES, pH 7.35, containing 0.2 mM PMSF, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 48 μg/ml aprotinin, 10 μg/ml leupeptin, 48 μg/ml soybean trypsin inhibitor, and 0.7 μg/ml pepstatin A (buffer A). Worms were sonicated at 4° with a titanium microtip probe for 3 min (1-sec pulses) at 150 W. Large preparations of worms (800 ml) were resuspended in buffer A in the proportions given above and were disrupted in a Manton-Gaulin homogenizer. Comparable [^{3}H]IVM-binding activity was obtained using either disruption technique. Disrupted worms were centrifuged at 1000 × *g* for 3 min to clear cuticle debris, and the supernatant was centrifuged at 28,000 × *g* for 1 hr. The 28,000 × *g* membrane pellet was resuspended in buffer A, in ground glass tissue homogenizers, and dialyzed for 12 hr against 50 mM HEPES, pH 7.4, with 0.2 mM PMSF, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, and 0.1 mM EDTA (buffer B). The dialyzed membrane homogenate was frozen in a dry ice/acetone bath and stored at −70° for subsequent assays, without loss of activity.

Detergent solubilization of the *C. elegans* IVM binding site. For all experiments except that performed in Fig. 1, *C. elegans* membrane homogenates were treated with 30 mM NOG at a final protein concentration of 2 mg/ml. The detergent-tissue suspension was incubated for 1 hr at 4°, with gentle rocking, and centrifuged for 1 hr at 100,000 × *g*. The pellet was resuspended by homogenization in buffer B for binding assays. The supernatant or NOG-soluble extract was frozen rapidly in a dry ice/acetone bath and stored at −70°, with no loss in IVM-binding activity over a period of 1 year. Detergent was not removed for binding assays except for the experiment in Fig. 1, where the supernatant was dialyzed against buffer B before IVM binding.

[^{3}H]IVM binding assay. [^{3}H]IVM binding was determined using a modification of a filtration assay previously described (15). [^{3}H]IVM was used at a final concentration of 1.3 nM, except for saturation binding studies and competition studies. Approximately 150–200 μg of membrane or NOG-soluble protein were added to tubes containing a 1-ml sample of [^{3}H]IVM, in either the presence (nonspecific binding) or absence (total binding) of 1 μM unlabeled IVM. Specific binding of IVM was calculated as the difference between total and nonspecifically bound and was linear from 75 to 300 μg/ml levels of both membrane and NOG-soluble protein. Assays were performed in buffer B with 0.02% TX-100 at 22° for 1 hr, except where noted. Samples were filtered through Whatman GF/B glass fiber filters, which were soaked for at least 20 min at 4° with 0.3% PEI and 0.25% TX-100. Filters were washed by vacuum filtration with three 5-ml aliquots of ice-cold 0.5% TX-100, and the radioactivity was measured in 5 ml of Aquasol-2 (Dupont) by liquid scintillation counting, with 64% efficiency of counting.

Preparation of lipid vesicles. Total lipid was extracted from *C. elegans* membranes by chloroform/methanol extraction, as previously described (18). Lipid vesicles were prepared essentially as described previously for other lipid sources (19), with the following modifications. Dried phospholipids were mixed with distilled H_2O at 50 mg/ml and immediately sonicated in a bath sonicator (Laboratory Supplies Co., Hicksville, NY) until the solution cleared, approximately 10 min. Approximately 100 μg of NOG-soluble protein were mixed with 1 mg of phospholipid vesicles at 22° for 15 min and diluted 4-fold to 1.5 mM NOG before the addition of [^{3}H]IVM.

Protein assays. Protein determinations were performed using the enhanced protocol of the Pierce bicinchoninic acid protein assay reagent. Bovine serum albumin was used as a standard.

Column chromatography of the NOG-soluble IVM binding site. Detergent extracts were filtered through a Millipore Millex-GV 0.22-μm filter unit immediately before chromatography. Protein applied to a Superose 6 HR 10/30 FPLC column (Pharmacia) was concentrated 18-fold, by ultrafiltration on an Amicon YM30 filter, and incubated at 4° for 3 hr with 0.2% TX-100 in buffer B. A 0.2-ml aliquot containing 2.5 mg of protein was applied to a preequilibrated column and eluted in 0.2% TX-100 in buffer B at a flow rate of 0.5 ml/min. Fractions (1.0 ml) were collected and assayed for specific IVM binding. The molecular weight standard proteins were thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 44,000), and myoglobin (M_r 17,000) (Bio-Rad), in addition to blue dextran. Blue dextran was used to determine the column void volume.

A 10-ml aliquot of NOG-soluble protein (2.8 mg) was equilibrated in 25 mM NOG in buffer B and applied to the anion exchange FPLC column Mono Q HR 5/5 (Pharmacia). The column was washed with 25 mM NOG in buffer B, and a 0–0.5 M linear NaCl gradient was applied at a flow rate of 1.0 ml/min. Fractions (2.0 ml) were collected and assayed for specific IVM binding. Fractions from a Mono Q HR 5/5 column that contained IVM-binding activity (6.7 mg) were pooled, equilibrated in 25 mM NOG in buffer B, and applied to a Mono P HR 5/20 FPLC (Pharmacia) column. A linear pH gradient was generated by elution of the column with 50 ml of a 1/10 dilution of polybuffer 74 (Pharmacia), pH 4.0, containing 25 mM NOG, 0.2 mM PMSF, 0.7 μg/ml pepstatin, and 0.5 μg/ml leupeptin, at a flow rate of 1.0 ml/min. After the pH gradient, additional protein was eluted from the column with starting buffer containing 0.5 M NaCl. Fractions (3.0 ml) were collected and a 200-μl sample was assayed for specific IVM binding.

Results

Solubilization of the IVM binding site of *C. elegans*. To further characterize the *C. elegans* high affinity IVM binding site (15), we attempted to detergent solubilize the IVM-binding activity from *C. elegans* membranes. A preparation of *C. elegans* membranes was treated with various amounts of the nonionic detergent NOG (Fig. 1). High speed centrifugation was used to separate the soluble and particulate fractions. After treatment with increasing concentrations of NOG, the amount of specific IVM binding in the pellet decreased, with simultaneous appearance of the activity in the supernatant. Release of the IVM-binding activity from the membranes was dependent on detergent treatment, because salt (1.0 M NaCl) or high and low pH (pH 8–5) treatment did not release IVM-binding activity from the membrane pellet (data not shown). Solubility of the detergent-extracted activity was verified by retention of binding activity in the filtrate after filtration through a 0.22-μm filter, as well as by chromatography on size exclusion matrices (see below). Optimum solubilization of the IVM-binding activity was achieved at 30 mM NOG, with loss of approximately 50% of the total activity and a slight increase in specific activity (Fig. 1, Table 1). Further titration of NOG (20, 25, 35, 45, 55, or 70 mM) did not improve solubilization. Changes in pH, salt, or temperature during NOG solubilization did not affect the yield of IVM-binding activity. The detergents CHAPS and octyl-β-D-thioglucopyranoside and the soybean phospholipid azolectin, in conjunction with NOG, did not improve solubilization.

For subsequent experiments, it was important to examine the stability of the IVM-binding activity. The NOG-soluble IVM-binding activity was stable, with retention of approximately 90% activity, when stored at either −70° for 1 year or 4° for 12 hr. Treatment of the NOG-soluble protein with salt (0.5 M NaCl), low pH (pH 4.0), or sulfhydryl-reducing agents (500 mM dithiothreitol) before addition of [^{3}H]IVM did not alter the binding activity.

A glass fiber filter assay was developed to assay specific [^{3}H]IVM binding to NOG-soluble extracts. Filtration of the NOG-soluble protein-[^{3}H]IVM complex on PEI-treated filters enabled the separation of free [^{3}H]IVM from [^{3}H]IVM bound to protein. Although PEI treatment was not necessary for membrane trapping on filters, it was a prerequisite for the binding of NOG-soluble protein on the filters (data not shown). Filtration of [^{3}H]IVM alone through PEI-treated filters followed by TX-100 washes resulted in no entrapment of [^{3}H]IVM. We also examined polyethylene glycol precipitation (20) for separating unbound and bound [^{3}H]IVM and found unacceptable levels of nonspecifically bound [^{3}H]IVM in the polyethylene glycol precipitate (data not shown).

To further optimize IVM binding to the NOG-soluble protein, we examined the effect of changes in pH, salt, temperature, and detergent and lipid composition in the assay mixture. Changes in salt and temperature did not increase IVM binding. A pH profile of binding was performed, and optimum binding was found at pH 5.5, with approximately 20% more specifically bound IVM than that observed at pH 7.4. However, at pH 5.5 we occasionally observed unacceptable levels of nonspecific [^{3}H]IVM binding, especially after column chromatography. All IVM binding studies were, therefore, conducted at pH 7.4. Changes in detergent composition had a pronounced effect on IVM binding. The binding of IVM to the solubilized protein was optimal at 5 mM NOG and 0.02% TX-100 (Table 2), even though the IVM-binding activity was extracted from membranes with 30 mM NOG. Therefore, for optimum IVM binding it was necessary to dilute the NOG-soluble extract to 5 mM NOG, which is below the critical micelle concentration of 25–30 mM for this detergent (21). The addition of TX-100 in the binding assay resulted in a dramatic decrease in nonspecific IVM binding, from 36% to 6% of total bound IVM (Table 2). To determine whether the IVM binding site bound IVM more

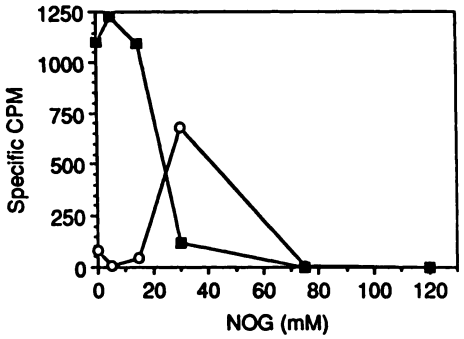

Fig. 1. Solubilization of the IVM-binding activity from *C. elegans* membranes. Membrane protein (1 mg) was treated with various concentrations of NOG and centrifuged for 1 hr at 100,000 × *g*. The pellet (■) and supernatant (○) were assayed in triplicate for specific IVM binding.

TABLE 1

K_D and B_{max} values for [^{3}H]IVM binding to *C. elegans* tissue

C. elegans protein from sonicated worms (1000 × *g* supernatant), membranes (28,000 × *g* pellet), or NOG-solubilized extracts (100,000 × *g* supernatant) was assayed for [^{3}H]IVM (0.1–2.6 nM) binding in the presence or absence of 1 μM unlabeled IVM. Values represent the mean ± standard error for two to six independent experiments.

Source of *C. elegans* tissue	K_D	B_{max}
	nM	*pmol/mg of protein*
Sonicated supernatants	0.50 ± 0.25	0.66 ± 0.23
Membrane pellets	0.11 ± 0.04	0.54 ± 0.04
NOG-soluble supernatants	0.20 ± 0.04	0.66 ± 0.23

TABLE 2

Assay conditions for the NOG-solubilized *C. elegans* IVM binding site

IVM binding assays were performed at 22° for 1 hr in 50 mM HEPES, pH 7.35. Specific IVM binding was calculated as the difference between total [^{3}H]IVM bound and the amount bound in the presence (nonspecific binding) of 1 μM unlabeled IVM. All samples used to determine the 100% IVM binding values were assayed in 5 mM NOG and 0.02% TX-100. The final detergent concentration in lipid reconstitution assays was 1.5 mM NOG. The source of purified protein was the pooled peak IVM-binding activity from a Superose 6 HR 10/30 FPLC column, as shown in Fig. 5. Crude protein represents the high speed (100,000 × *g*) supernatant from NOG-treated membranes. All assays were performed in duplicate.

Assay composition	Bound IVM		IVM binding[a]
	Specific	Nonspecific	
	fmol/mg		%
Detergent			
5 mM NOG + 0.02% TX-100	593	40	100[b]
30 mM NOG	9	32	2[b]
5 mM NOG	347	198	59[b]
Lipid			
Azolectin vesicles	62	16	25[c]
C. elegans vesicles + purified protein	477	161	150[d]
C. elegans vesicles + crude protein	404	98	116[e]

[a] The specific activities (fmol/mg) of the starting protein samples were as follows: [b] 593, [c] 251, [d] 315, and [e] 348.

effectively if the NOG-soluble protein was reconstituted in lipid vesicles, reconstitution experiments were performed. Reconstitution by detergent dilution into azolectin vesicles appeared to inhibit IVM binding, whereas reconstitution into *C. elegans*-derived lipid vesicles showed approximately 16–50% greater IVM binding (Table 2). The lipid-enhanced IVM binding was observed to a greater extent in protein preparations that were partially purified by size exclusion chromatography (Table 2). Because lipid reconstitution was not required to detect IVM binding, it was not used for further assays presented in this report.

Equilibrium binding analysis. *C. elegans* membranes and NOG-soluble protein extracts were analyzed by equilibrium saturation binding with various concentrations of [^{3}H]IVM (Fig. 2). Scatchard analysis of the saturation binding data shows a linear plot for both membranes (Fig. 2A) and NOG-soluble extracts (Fig. 2B). The affinity of binding, K_D, of the membranes and NOG-soluble extracts was 0.138 nM and 0.179 nM, respectively. The capacity of IVM binding, B_{max}, was 0.57 pmol/mg of membrane protein and 0.82 pmol/mg of NOG-soluble protein. Nonspecific binding increased with increasing [^{3}H]IVM concentration and was consistently higher in membrane assays (15%), compared with NOG-soluble extracts (7%), at 2.6 nM [^{3}H]IVM. Analysis of several IVM binding experiments showed an average K_D of 0.11 ± 0.04 nM and 0.20 ± 0.04 nM, with a B_{max} of 0.54 ± 0.04 pmol/mg and 0.66 ± 0.23 pmol/mg, for membranes (five experiments) and NOG-soluble extracts (five experiments), respectively (Table 1).

Binding kinetics. The rates of association and dissociation of [^{3}H]IVM with membranes and NOG-soluble extracts are shown in Fig. 3. The IVM binding to membranes reached 100% or saturation at 5 min, whereas IVM binding to NOG-soluble extracts required 30 min to reach saturation. The association rate was determined using the second-order rate equation $\ln(B_o - x/A_o - x) = (B_o - A_o)k_{+1}t$, where B_o is the [^{3}H]IVM concentration at $t = 0$ (1.3 nM) and A_o is the concentration of binding sites at $t = 0$, or the B_{max} as determined by saturation binding studies. For these analyses, the B_{max} was 0.114 nM and 0.075 nM for membranes and NOG-soluble extracts, respectively. The concentration of [^{3}H]IVM used for these studies was 1.3 nM, a saturating amount of ligand (see Fig. 2). The value x is the amount of [^{3}H]IVM and binding site that have reacted, or the amount bound. The k_{+1} was calculated to be 0.074 nM^{-1} min^{-1} for membranes and 0.053 nM^{-1} min^{-1} for NOG-soluble extracts. The mean value of three experiments gave a k_{+1} value of 0.044 ± 0.013 nM^{-1} min^{-1} for NOG-soluble extracts.

C. elegans membranes and NOG-soluble extracts form high affinity complexes with IVM that exhibit slow dissociation rates after 60 min of binding to [^{3}H]IVM (Fig. 3B). Using the data obtained from Fig. 3B, the dissociation rate was determined using the equation $\ln(B/B_o) = -k_{-1}t$, where B is the [^{3}H]

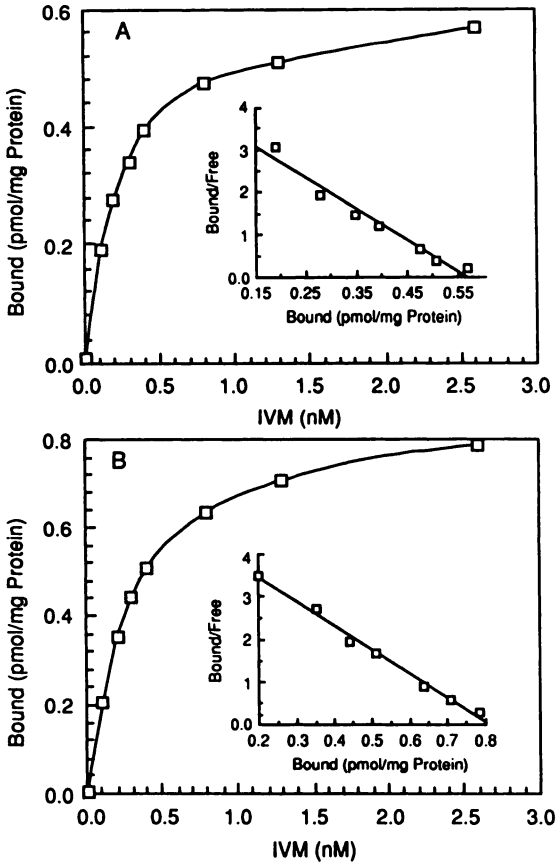

Fig. 2. Saturation binding of IVM to membranes (A) or NOG-soluble protein extracts (B) was performed with the indicated concentrations of [^{3}H]IVM. Samples of 200 μg of protein were assayed in triplicate for specific IVM binding. Bound represents the pmol of [^{3}H]IVM trapped on the glass fiber filters/mg of protein in the assay. Free [^{3}H]IVM is the difference between the input [^{3}H]IVM and the amount bound/sample. Analysis of these data indicates a K_D of 0.14 nM and 0.18 nM, with a B_{max} of 0.57 pmol/mg and 0.82 pmol/mg, for membranes and NOG-soluble extracts, respectively.

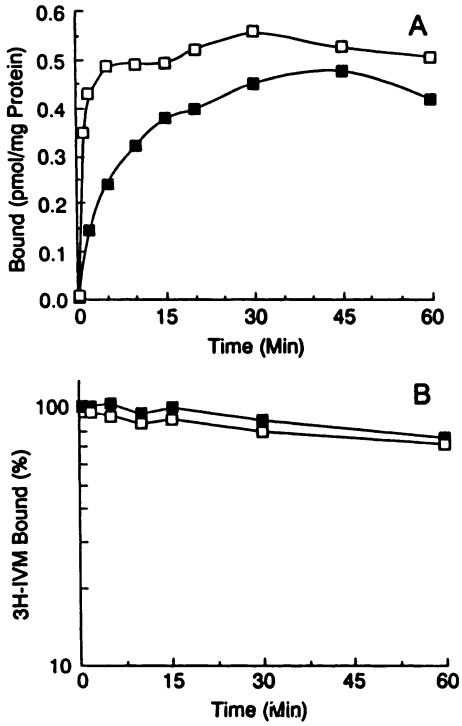

Fig. 3. Rates of association and dissociation of IVM with *C. elegans* membranes or NOG-soluble protein. A, Specific binding of IVM to 150 μg of soluble protein (■) and 200 μg of membrane protein (□) was measured as a function of time. B, The dissociation kinetics of IVM and membranes (□) or NOG-soluble protein (■) was determined by binding 1.3 nM [^{3}H]IVM to 150 μg of tissue for 60 min at 22°. Unlabeled IVM (1 μM) was then added, and the reaction was terminated at the indicated times. The amount of [^{3}H]IVM bound at $t = 0$ was 0.076 pmol and 0.046 pmol for membranes and soluble extracts, respectively. All data points were assayed in duplicate.

IVM bound and B_o is the amount bound at $t = 0$. The calculated rate constant for dissociation was $k_{-1} = 0.006$ min^{-1} for membranes and 0.005 min^{-1} for NOG-soluble extracts. In our dissociation experiments carried out after 1, 2, 3, or 5 min of binding, the k_{-1} values were 0.008 ± 0.002 min^{-1} for membranes (six experiments) and 0.008 ± 0.003 min^{-1} for NOG-soluble extracts (five experiments) (data not shown). Using the relationship $K_D = k_{-1}/k_{+1}$ the K_D values were determined to be 0.08 nM and 0.11 nM for membranes and NOG-soluble extracts, respectively. These values compare favorably with the apparent K_D as determined by Scatchard analysis (Table 1) of 0.11 ± 0.04 nM and 0.20 ± 0.04 nM for membranes and NOG-soluble extracts, respectively.

Stereospecificity of IVM binding. The stereospecificity of [^{3}H]IVM binding to the membrane and NOG-soluble binding site was tested by competing for the binding of [^{3}H]IVM with three AVM analogs (Fig. 4). The analogs used, with the K_i values for membranes and NOG-soluble extracts, respectively, were 22,23-dihydro-AVM B_{1a} monosaccharide, 2.95 nM and 4.20 nM; 22,23-dihydro-AVM B_{1a} aglycone, 18.81 nM and 31.25 nM; and 3,4,8,9,10,11,22,23-octahydro B_1 AVM, >350 nM and >500 nM. The measured K_i for IVM was 0.29 nM for both membranes and NOG-soluble extracts. Several electrophysiology experiments have indicated that IVM interacts with the GABA-gated chloride channel in invertebrates (10, 12). Although the membrane-bound IVM binding site was shown not to bind GABA or several GABA agonists and antagonists (15), we measured the effect of GABA on [^{3}H]IVM binding to the NOG-soluble extracts. No competition of IVM binding was seen with GABA at concentrations up to 10 μM (Fig. 4).

Biochemical characterizations. The biochemical properties of the NOG-soluble *C. elegans* IVM-binding protein were examined chromatographically (Fig. 5). The NOG-soluble IVM-binding activity was concentrated and chromatographed on the FPLC gel filtration column Superose 6, which is capable of separating molecules ranging from 5,000,000 to 1,000 Da (Fig. 5A). The IVM-binding activity was found in fractions 13–15, which represented approximately 44% of the applied activity. Fraction 14 eluted at an apparent M_r of approximately 340,000 in the presence of TX-100, which in the absence of protein has an estimated micell size of 90,000 Da (21).

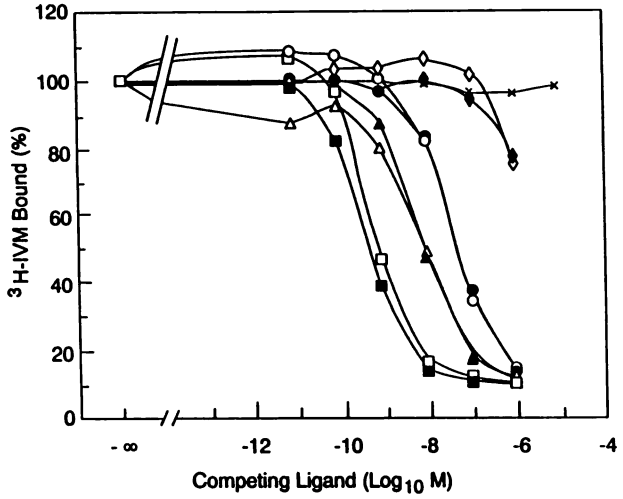

Fig. 4. Inhibition of specific IVM binding to *C. elegans* membranes or NOG extracts by AVM analogs and GABA. Protein (150 μg) from membranes (*open symbols*) or NOG-treated extracts (*closed symbols*, ×) were incubated with 0.2 nM [^{3}H]IVM for 60 min at 22°, in the presence of the indicated concentrations of IVM (*squares*), 22,23-dihydro-AVM B_{1a} aglycone (*circles*), 22,23-dihydro-AVM B_1 monosaccharide (*triangles*), 3,4,8,9,10,11,22,23-octahydro B_1 AVM (*diamonds*), or GABA (×). All data points were assayed in triplicate.

NOG-soluble extracts were applied to the anion exchange FPLC column Mono Q (Fig. 5B). IVM-binding activity bound to the column at pH 7.4 and could be eluted at approximately 0.2 M NaCl. To measure the pI of the IVM binding site, Mono Q-purified NOG-soluble extracts were chromatographed on the chromatofocusing FPLC column Mono P (Fig. 5C). The IVM-binding activity bound to the column and was eluted with a linear polybuffer gradient from pH 7.4 to 4.0. The IVM-binding activity eluted in a major peak at pH 4.2–4.0, with a minor amount of activity in fractions eluting at pH 6.0. This indicated that the IVM-binding protein has a pI of 4.0. The yield of IVM-binding activity from the chromatofocusing Mono P column was poor, with approximately 30% of the applied activity in fractions 20 and 21 (approximately 0.3 pmol/mg of protein). To determine whether a component necessary for IVM binding had been removed, fractions 20 and 21 were mixed with the remaining fractions and reassayed for IVM binding activity. No enhancement of binding was observed in this experiment.

Discussion

We have used the nematode *C. elegans* as a model system to study IVM action because it is extremely sensitive to IVM *in vivo* (15), large quantities of worms are readily available, and the biology of the organism is well defined (22). In addition, it has been shown that there is a direct correlation between the *in vivo* potency of AVM derivatives and the affinity of binding in *C. elegans* membrane preparations (15). We have searched for a high affinity IVM binding site in the parasitic nematode *Haemonchus contortus*, using the same protocols described in this study. IVM binding in the membranes or NOG-soluble extracts of the infectious L3 stage larvae was not observed, but a small amount of specific IVM binding in membrane extracts of the adult stage was found (data not shown). The characterization and ultimate purification of the *C. elegans* IVM binding site will provide more sensitive tools with which to study the mode of action of IVM in parasitic nematodes.

In this study we show that a membrane-bound, high affinity, IVM binding site from *C. elegans* can be solubilized with the nonionic detergent NOG. The NOG-solubilized and membrane forms of the IVM binding site exhibit several common properties; 1) the membrane-bound and NOG-soluble IVM binding sites exhibit the same pharmacological properties for AVM derivatives; 2) the high affinity for IVM is retained; and 3) the kinetic properties of IVM binding are unaltered. We conclude from these observations that a high affinity membrane-bound IVM binding site has been detergent solubilized and that this treatment did not greatly alter the properties of the binding protein.

IVM binding to NOG-soluble protein was assayed by trapping of the IVM-binding protein complex on PEI-treated glass fiber filters. Treatment of the filters with the cationic polymer PEI results in a positive charge that binds negatively charged soluble receptors (23). We believe that the NOG-soluble IVM binding site is an acidic protein(s), because it binds to PEI-treated filters and anion exchange columns at pH 7.35 and has a pI of 4.0. Gel filtration of the IVM binding site in the presence

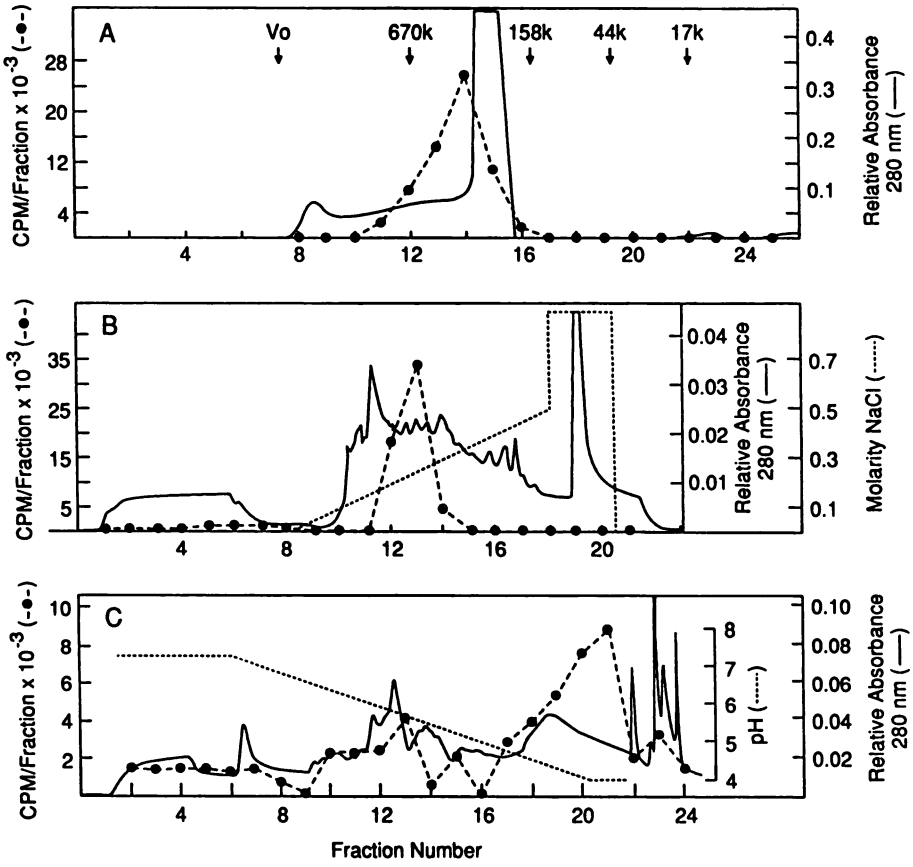

Fig. 5. Column chromatography of the NOG-soluble IVM binding site. A, Size exclusion chromatography of the NOG-soluble IVM binding site on a Superose 6 FPLC column. Column fractions (1 ml) were collected and assayed for specific IVM binding. The elution profile of molecular weight markers is noted at the *top*. B, Anion exchange chromatography of the NOG-soluble IVM binding site on a Mono Q HR 5/5 FPLC column. Fractions (2.0 ml) were collected and assayed for specific IVM binding. C, Chromatofocusing of the NOG-soluble IVM binding site. Pooled fractions containing IVM-binding activity from a Mono Q column were applied to the chromatofocusing FPLC column Mono P HR 5/20. A linear pH gradient was generated, and fractions (3.0 ml) were collected and assayed for specific IVM binding. All column samples were measured in duplicate. The cpm bound/fraction represents the total amount of IVM-binding activity in the column fraction.

of the detergent TX-100 predicts an apparent M_r of 340,000, which may indicate that the binding site exists as a complex of several polypeptides, as found for the $GABA_A$ receptor (for review, see Ref. 24) and the nicotinic acetylcholine receptor (for review, see Ref. 25).

Optimum IVM binding was observed when the NOG-soluble extracts were diluted to 5 mM NOG, which is below the critical micelle concentration of NOG (21). We have observed that at 5 mM NOG the IVM-binding activity is found in the pellet after high speed centrifugation (data not shown). Thus, at this detergent concentration it is possible that vesicles are formed that contain lipid and protein. In addition, stimulation of IVM binding was observed when NOG-soluble protein that had been partially purified by gel filtration was reconstituted in *C. elegans* lipid vesicles. Although these results are not conclusive, they may indicate that a minimum number of lipids are required for optimum IVM binding, as has been observed for the nicotinic acetylcholine receptor (26) and the D1 dopamine receptor (27).

We have observed that both *C. elegans* membranes and NOG-soluble extracts form high affinity complexes with IVM that exhibit slow dissociation rates of 0.006 and 0.005 min^{-1}, respectively. The binding of IVM to the membrane-bound high affinity *C. elegans* binding site was previously reported to be a two-step process, requiring first the formation of a readily dissociable complex, followed by the formation of a second, more slowly dissociating, complex (15). We were unable to verify this property of IVM binding with our tissue preparations. This difference may be attributed to the different methods used to disrupt the worms or to the addition of protease inhibitors in our disruption media (15).

It has been proposed that the potency of IVM in the binding assays may indicate the relative potency *in vivo* (15). The EC_{50} of IVM is approximately 7–15 nM in a *C. elegans* liquid motility assay (data not shown) (15) and 3 nM on Petri dishes with *E. coli* as a food source (data not shown). We tested the potency of three AVM analogs to compete with [^{3}H]IVM binding to the NOG-soluble binding site. These specific AVM analogs were chosen because we have observed that they exhibit different potencies *in vivo* with *C. elegans*, with EC_{50} values of 32 nM, 260 nM, and >1.1 μM for 22,23-dihydro-AVM B_{1a} monosaccharide, 22,23-dihydro-AVM B_{1a} aglycone, and 3,4,8,9,10,11,22,23-octahydro B_1 AVM, respectively (data not shown). In our studies, competition of IVM binding to the NOG-soluble binding site with these AVMs showed inhibitory constants that reflected the *in vivo* potencies of the drugs.

The information obtained from this study will facilitate subsequent purification and thus further understanding of the nature of the *C. elegans* IVM binding site. The solubilization and biochemical characterization of other drug-binding proteins, such as the $GABA_A$ receptor, which binds the benzodiazepines (28–30), have contributed to our knowledge of drug action and may lead to the development of new pharmaceuticals.

Acknowledgments

We thank Dr. Helmut Mrozik for supplying and verifying the AVM derivatives; Dr. K. Gbewonyo and L. Lister for preparation of the 150-liter *C. elegans* cultures; Dr. W. Shoop for supplying *H. contortus*; and Drs. J. Arena, R. McKernan, W. Shoop, A. Sidhu, and J. Schaeffer for critical review of this manuscript.

References

1. Campbell, W. C., M. H. Fisher, E. O. Stapley, G. Albers-Schonberg, and T. A. Jacob. Ivermectin: a potent new antiparasitic agent. *Science (Washington D. C.)* **221**:823–828 (1983).

2. Campbell, W. C. *Ivermectin and Abamectin*. Springer-Verlag, New York (1989).
3. Chabala, J. C., H. Mrozik, R. L. Tolman, P. Eskola, A. Lusi, L. H. Peterson, M. F. Woods, and M. H. Fisher. Ivermectin, a new broad-spectrum antiparasitic agent. *J. Med. Chem.* **23**:1134–1136 (1980).
4. 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).
5. Duce, I. R., and R. H. Scott. Actions of dihydroavermectin B_{1a} on insect muscle. *Br. J. Pharmacol.* **85**:395–401 (1985).
6. 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).
7. Lees, G., and D. J. Beadle. Dihydroavermectin B1: actions on cultured neurones from the insect central nervous system. *Brain Res.* **366**:369–372 (1986).
8. 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).
9. Scott, R. H., and I. R. Duce. Effects of 22,23-dihydroavermectin B_{1a} on locust (*Schistocerca gregaria*) muscles may involve several sites of action. *Pestic. Sci.* **16**:599–604 (1985).
10. 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 motoneurons in *Ascaris*. *Proc. Natl. Acad. Sci. USA* **77**:6211–6215 (1980).
11. Holden-Dye, L., G. M. Hewitt, K. T. Wann, P. Krogsgaard-Larsen, and R. J. Walker. Studies involving avermectin and the 4-aminobutyric acid (GABA) receptor of *Ascaris suum* muscle. *Pestic. Sci.* **24**:231–245 (1988).
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. 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).
14. Abalis, I. M., and A. T. Eldefrawi. [^{3}H]Muscimol binding to a putative GABA receptor in honey bee brain and its interaction with avermectin B_{1a}. *Pestic. Biochem. Physiol.* **25**:279–287 (1986).
15. 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).
16. Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **77**:71–94 (1974).
17. 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).
18. Palmer, F. B. S. C. The extraction of acidic phospholipids in organic solvent mixtures containing water. *Biochim. Biophys. Acta.* **231**:134–144 (1971).
19. Hale, C. C., R. S. Slaughter, D. C. Ahrens, and J. P. Reeves. Identification and partial purification of the cardiac sodium-calcium exchange protein. *Proc. Natl. Acad. Sci. USA* **81**:6569–6573 (1984).
20. Gavish, M., R. R. Goodman, and S. H. Snyder. Solubilized adenosine receptors in the brain: regulation by guanine nucleotides. *Science* (Washington D. C.) **215**:1633–1635 (1982).
21. Hjelmeland, L. M., and A. Chrambach. Solubilization of functional membrane proteins. *Methods Enzymol.* **104**:305–318 (1984).
22. Wood, W. B. Introduction to *C. elegans* biology, in *The Nematode Caenorhabditis elegans* (W. B. Wood, ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1–16 (1988).
23. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
24. Olsen, R. W., and A. J. Tobin. Molecular biology of GABA A receptors. *FASEB J.* **4**:1469–1480 (1990).
25. McCarthy, M. P., J. P. Earnest, E. F. Young, S. Choe, and R. M. Stroud. The molecular neurobiology of the acetylcholine receptor. *Annu. Rev. Neurosci.* **9**:383–413 (1986).
26. Jones, O. T., J. H. Eubanks, J. P. Earnest, and M. G. McNamee. A minimum number of lipids are required to support the functional properties of the nicotinic acetylcholine receptor. *Biochemistry* **27**:3733–3742 (1988).
27. Sidhu, A. Solubilization and reconstitution of the D-1 dopamine receptor: potentiation of the agonist high affinity state of the receptor. *Biochemistry* **27**:8768–8776 (1988).
28. Mohler, H., M. K. Battersby, and J. G. Richards. Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc. Natl. Acad. Sci. USA* **77**:1666–1670 (1980).
29. Stephenson, F. A., A. E. Watkins, and R. W. Olsen. Physiochemical characterization of detergent-solubilized *gamma*-aminobutyric acid and benzodiazepine receptor proteins from bovine brain. *Eur. J. Biochem.* **123**:291–298 (1982).
30. Sigel, E., F. A. Stephenson, C. Mamalaki, and E. A. Barnard. A *gamma*-aminobutyric acid/benzodiazepine receptor complex of bovine cerebral cortex. *J. Biol. Chem.* **258**:6965–6971 (1983).

Send reprint requests to: Dr. D. F. Cully, Merck & Co., Box 2000 R80T-124, Rahway, NJ 07065.