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The Levamisole Receptor, a Cholinergic Receptor of the Nematode *Caenorhabditis elegans*

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SUMMARY

We describe a glass fiber filter binding assay for the levamisole receptor, a putative acetylcholine receptor of the nematode *Caenorhabditis elegans*, and we show that receptor detected *in vitro* binds both levamisole derivatives and cholinergic agonists with the pharmacological specificity expected of the physiologically functional nematode receptor. The receptor is detected by the binding of tritiated *meta*-aminolevamisole ([^{3}H]MAL, 27 *Ci*/mmol). In extracts of the wild-type nematode, there is a saturable, high affinity binding activity for [^{3}H]MAL (K_d ~5–10 nM). Well fed wild-type worms contain as much as 3 fmol of high affinity binding activity per mg of extract protein (0.14 pmol/g of wet weight of worms) and dauer larvae, a special juvenile stage, contain as much as 15 fmol of activity per mg of protein. Specific binding activity per mg of protein is highest in larval stages and decreases severalfold in the adult worm. The rates of formation and dissociation of the [^{3}H]MAL-receptor complex are relatively slow (dissociation half-life, 17 min), in agreement with physiological studies of levamisole on *Ascaris* muscle strips. Levamisole derivatives and cholinergic agonists have the same relative potencies in inhibiting [^{3}H]MAL binding as they do in causing nematode muscle contraction. Vertebrate cholinergic antagonists do not inhibit [^{3}H]MAL binding, but several antagonists (mecamylamine, α-bungarotoxin, and cobra venom) potentiate the binding of [^{3}H]MAL and can be used to demonstrate more clearly the presence of a second, lower affinity binding activity whose ligand-binding affinity is also potentiated by these agents. Both high and low affinity wild-type binding components are missing in the extremely levamisole-resistant mutant *unc-74(x19)*.

In this paper we describe a binding assay for an apparent nematode acetylcholine receptor that is now amenable to a powerful combination of classical and molecular genetic analysis. Receptor mutants of the nematode *Caenorhabditis elegans* can be isolated by selection for resistance to the toxic muscle-hypercontracting effects of the nicotine-like drug levamisole (1–3). The most resistant mutants can arise by mutation of any one of seven genes, and all share a similar phenotype of uncoordinated motor behavior, most severe early in larval life, and have increased resistance to all cholinergic agonists that cause muscle contraction of a normal worm. The pharmacological and developmental phenotypes of the mutants suggest that the affected receptor is most important in the nervous system of juveniles and may be but one of several cholinergic receptor types present in the nematode. The nonessentiality of the receptor greatly facilitates genetic manipulation and, from the number and characteristics of mutated genes that give rise to drug resistance, it appears likely that the genes identified may encode not only structural peptides for the receptor but possibly also functions needed in either receptor regulation or processing. Because most nematode genes identifiable by classical genetics can now be cloned by transposon tagging (4–6), and because transformation of DNA back into an intact organism is also possible (7), analysis of receptor gene function can be prospectively extended to the DNA sequence level and, given the nonessentiality of receptor function, receptor gene expression should be manipulable within the developmental context of the intact organism.

To characterize the receptor molecules present in wild-type and levamisole receptor mutants, a binding assay is needed. The agents most useful for the assay of vertebrate cholinergic receptors, α-bungarotoxin, benzyltrimethylammonium, and 3-quinuclidinyl benzilate, do not have a significant pharmacological effect on nematodes (2).

We report here that [^{3}H]MAL, a much more potent derivative of the mutant selective agent levamisole, can be used to detect a saturable, high affinity levamisole binding activity in *C. elegans* extracts. The properties characterizing this binding activity, including its stereospecificity and preference in binding levamisole derivatives and cholinergic agonists, are similar to what might be expected from the *in vivo* pharmacological effects of these drugs and support a cholinergic receptor function for the detected binding activity. Known vertebrate cho-

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ABBREVIATIONS: MAL, *meta*-aminolevamisole; POPOP, 1,4-bis[5-phenyl-2-oxazolyl]benzene.

linergic antagonists do not inhibit [^{3}H]MAL binding by the nematode receptor, but several antagonists, notably mecamylamine, α-bungarotoxin, and venom of the cobra *Naja naja kaouthia*, allosterically activate [^{3}H]MAL binding.

In a separate paper, we will report our findings that mutants of the seven genes associated with extreme resistance to levamisole are all deficient or distinctly altered in levamisole binding activity.

Experimental Procedures

Materials. Acetylthiocholine chloride, atropine sulfate, bethanecol, carbachol, choline chloride, decamethonium bromide, 5,5′-dithiobis(2-nitrobenzoic acid), eserine sulfate, hexamethonium bromide, mecamylamine hydrochloride, muscarine hydrochloride, 3-*N*-morphilinopropanesulfonic acid, nicotine, procaine hydrochloride, tricaine, Triton X-100, and venom of the cobra *Naja naja kaouthia* were obtained from Sigma Chemical Co.; dimethylphenylpiperazinium iodide, pentolinium tartrate, and phenyltrimethylammonium iodide were from Aldrich Chemical Co.; and α-bungarotoxin and *d*-tubocurarine chloride were from Boehringer Mannheim Biochemicals. Whatman GF/C glass fiber filters and Prosil-28 were purchased from American Scientific Products. Ficoll 400 was a product of Pharmacia Fine Chemicals. The following chemicals were obtained as gifts from the respective companies: MAL, levamisole hydrochloride, and the various substituted levamisole derivatives, from American Cyanamid; quinuclidinyl benzilate, from Hoffman La-Roche; dexamisole hydrochloride, from Janssen Pharmaceutica; morantel tartrate, from Pfizer; and aldicarb, from Union Carbide. Nitex filters were provided by Tekto.

Strains and culture conditions. The wild-type strain of *C. elegans* used was the Bristol strain (1). The levamisole-resistant mutants ZZ19 [*unc-74(x19)*] and ZZ1006 [*lev-1(x21) x1006rt*] were isolated by us (3).

Worms were grown in mass culture at 20–22° employing the media of Sulston and Brenner (8). Worms were settled overnight at 4° and then cleaned by sedimentation for 15 min at 300 × *g* through 15% (w/w) Ficoll 400, 0.1 M in NaCl, diluted with an equal volume of 0.1 M NaCl, and floated for 15 min at 300 × *g* on 35% (w/w) Ficoll 400, 0.1 M in NaCl, and then washed twice with 0.1 M NaCl, and stored frozen in liquid nitrogen. Worms that consisted of dauer larvae and starved early stage juveniles (L1–L2) were obtained from cultures starved for 1 week by flotation after mixing with 30% (w/v) sucrose followed by centrifugation of the collected fraction through 15% (w/w) Ficoll 400 and washing with NaCl.

To obtain worms of reasonably specific age, Petri plates (100 mm diameter) containing *Escherichia coli* grown on NGM medium (1) were seeded with 15 adults each and after 4 days at 20°, the progeny were collected by filtration (9). Adult worms were collected by retention on a 30 μm mesh Nitex filter, whereas the worms obtained after passage through both 30 μm and 20 μm mesh Nitex filters were mainly first stage larvae. Worms were cleaned and frozen as described above.

Preparation of [^{3}H]MAL. [^{3}H]MAL (27 Ci/mmol) was synthesized as previously described (10). For some experiments, as indicated, [^{3}H]MAL taken only from the peakmost fractions of a Florisil column was used. The use of a more select [^{3}H]MAL fraction appeared to affect only the amount of nonspecific binding measured.

Siliconization of filters. Whatman GF/C glass fiber filters (2.4 cm diameter) were soaked 5 min to a few hr in Prosil-28 diluted 1:100 with distilled water (100 filters/200 ml of solution). Filters were rinsed three times with water and dried 30 min at 120° or air-dried for 24 hr at room temperature.

Binding assay. Thawed worms were diluted less than 50% with the assay buffer, 10 mM 3-(*N*-morphilino)propanesulfonic acid, pH 6.8, 100 mM NaCl, 10 mM sodium azide, and passaged twice at 8000 psi through a French pressure cell chilled to 0°. An assay consisted of 30 μl of assay buffer containing [^{3}H]MAL, 10 μl of distilled water containing any necessary drug additions, and 60 μl of worm extract diluted with assay buffer to contain 0.6–1.0 mg of extract protein. Solutions containing [^{3}H]MAL in assay buffer were made up fresh prior to each assay as the binding activity of [^{3}H]MAL decays with a half-life of about a week in azide-containing buffer kept at 4°. Stock [^{3}H]-MAL solutions were kept in liquid nitrogen. After incubation on ice for the specified amount of time, each assay was terminated by dilution with 5 ml of *ice-cold* assay buffer (0.01%) in Triton X-100 (wash buffer) and immediately passed through a glass fiber filter that had previously been wetted in buffer (1%) in Triton X-100 and rinsed with 5 ml of wash buffer. The filter was then washed three times with 5 ml of *ice-cold* wash buffer, placed in a vial with 4 ml of scintillation fluid (composed of 15.2 g of 2,5-diphenyloxazole, 0.31 g of POPOP, 0.34 liter of water, 1.2 liters of Triton X-100, and 2.3 liters of toluene per 3.8 liters) and incubated 20 hr at 37° before counting for 10–20 min at an efficiency of 46%. Each assay point represents the mean difference between quadruplicate determinations of total and nonspecific binding. Nonspecific binding was determined by measuring binding activity in the presence of 10 or 20 μM unlabeled MAL or 0.5 mM dimethylphenylpiperazinium, a competitor found to be preferable in later experiments. For competitive inhibition studies, nonspecific binding was usually determined in the presence of 10 μM unlabeled MAL only. To show that a competitor inhibited the same binding activity that unlabeled MAL did, the amount of inhibition caused by the IC_{50} concentration of the competitor plus unlabeled MAL was shown to be the same as that caused by 10 μM unlabeled MAL alone. In the few instances where a drug affected the level of nonspecific [^{3}H]MAL binding or appeared to potentiate specific [^{3}H]MAL binding, nonspecific [^{3}H]MAL binding controls were always done with the indicated concentration of the drug present as well as 10 μM unlabeled MAL. Binding data for drug inhibition studies were analyzed after logit transformation using $\log_{10}$ (a Hill equation) by weighted linear regression (11), as necessitated by the transformation. Error in the estimation of IC_{50} values was determined by inverse regression (12). Untransformed binding data were analyzed by weighted nonlinear regression using the SAS Institute, Inc., program for nonlinear regression, NLIN (13), or the Biomedical Computer Program (BMDP) program for nonlinear regression, P3R (14).

Acetylcholinesterase was assayed spectrophotometrically using 1 mM acetylthiocholine chloride (15). Protein was determined by the Lowry method as modified by Dully and Grieve (16).

Results

Assay requirements. [^{3}H]MAL sticks to untreated glass fiber filters and the binding is not reduced by prefiltering the radioactive ligand through a glass fiber filter. Siliconization with Prosil-28 and inclusion of 0.01% Triton X-100 in the wash buffer reduced nonspecific binding to the filters 20-fold. Presoaking filters with 0.1 M levamisole or imidazole was not helpful. A small assay volume (100 μl) minimizes the amount of [^{3}H]MAL that must be passed through a filter. Sodium azide is used in the assay mix to inhibit a secondary, time-dependent uptake of [^{3}H]MAL, possibly an active transport process. Increasing salt concentration in the wash buffer only reduces specific binding and nonspecific binding by similar amounts. An assay pH of 6.8 appeared to provide a shallow optimum in the measurement of specific [^{3}H]MAL binding in the pH range between 6.0 and 7.5. When 1 mg of extract protein was assayed at 11 nM [^{3}H]MAL as described in Experimental Procedures, the input cpm was 32,000, total binding was 240 cpm, nonspecific binding 160 cpm, and background without extract 30 cpm. Specific binding was 80 cpm with a standard error of the difference of 6 cpm for quadruplicate assays counted 10 min each. At 11 nM [^{3}H]MAL, minimum assay sensitivity is thus about 4×10^{-16} mol. Nonspecific binding and assay error increased linearly with increasing [^{3}H]MAL concentrations.

Assays were done in the range of 0.6–1.0 mg of extract protein per assay without any correction for a slight nonlinearity (15% underestimate) in the amount of specific binding predicted at 1.0 mg of protein based on the assay of 0.6 mg of extract protein.

More than 99% of specific [^{3}H]MAL binding at 11 nM [^{3}H]MAL was pelletable after centrifugation for 30 min at 33,000 × *g* and 75% of the pelletable activity[1] was extractable into 1% Triton X-100, indicating that most specific [^{3}H]MAL binding is membrane bound. The same binding activity found in total homogenates was observed over the range of 0.9–104 nM [^{3}H]MAL upon dispersing and assaying the pelleted material. Assays of total homogenate were used throughout this work, however, because of greater reproducibility.

Extract lost little binding activity upon standing 24 hr on ice. During the assay itself, specific [^{3}H]MAL binding measured at 3.4 nM [^{3}H]MAL did not change significantly once equilibrium was reached for up to 6 hr of total incubation, implying stability of receptor and ligand under assay conditions.

Extract activity could be increased 40–50% by freezing and thawing five times in liquid nitrogen and was then stable for at least another eight freeze-thaw cycles. The activity increase was probably caused by aggregation of insoluble material, as the increase could be reversed by repassage through a French pressure cell and could then be regained by subsequent freeze-thawings.

Equilibrium binding of [^{3}H]MAL. The specific [^{3}H]MAL binding observed in extracts of both larval and adult nematodes showed saturable behavior with increasing [^{3}H]MAL concentration (Fig. 1A). The nonlinearity of Scatchard plots (Fig. 1B) can apparently be explained by the presence of three binding components. The levamisole receptor itself is composed of a high affinity and a low affinity saturable specific [^{3}H]MAL binding activity that can be copurified more than 10,000-fold.[2] The use of 10 μM unlabeled MAL in the determination of nonspecific binding causes a third component to be included in the measurement of total specific [^{3}H]MAL binding. This third, nonlevamisole receptor component, unlike the other two components, is not soluble in Triton X-100 and is unaffected in mutants having essentially no Triton X-100-extractable binding activity. Because the low affinity receptor sites bind [^{3}H]MAL on the order of 20–30 times weaker than the high affinity sites and because of the limiting amounts of receptor activity in extracts of well fed worms, we have chosen a model of receptor binding that consists of a high affinity saturable component (hyperbola) and a single linear nonsaturating low affinity component (line), subsuming both low affinity levamisole receptor and nonlevamisole receptor components into one parameter. We found later that the third component can be eliminated from the determination of specific binding by using 0.5 mM dimethylphenylpiperazinium as the unlabeled competitor instead of MAL. However, whether or not the third component is eliminated, the hyperbola and line model is the best and most complex model possible because neither low affinity component saturates within the range of the assay. Using this model, we find that the wild-type larval extract (Fig. 1A, *upper curve*) consists of 2.8 ± 0.3 fmol/mg protein of high affinity saturable specific [^{3}H]MAL binding activity with a dissociation

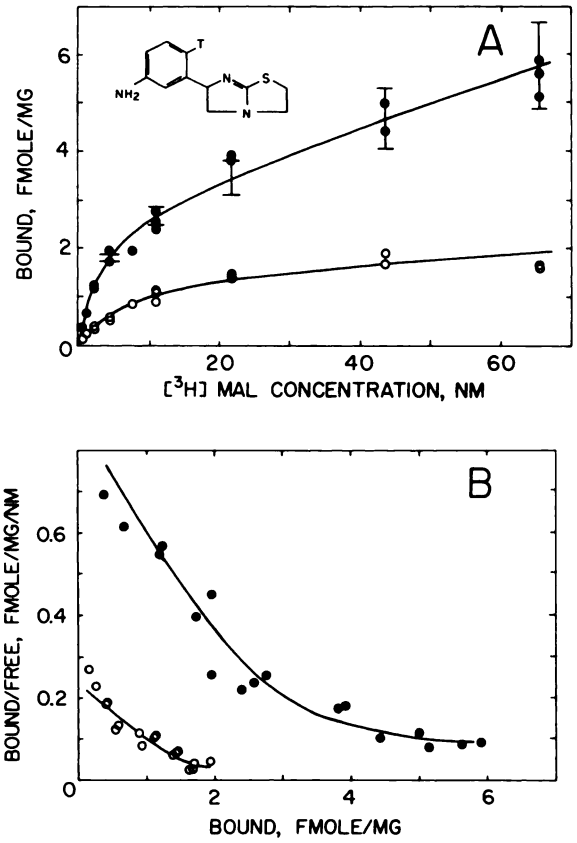

Fig. 1. Specific binding of [^{3}H]MAL to nematode tissue as a function of [^{3}H]MAL concentration. A. Five times frozen and thawed extract made from predominantly first stage larvae (●) and from adult nematodes (○), 1 mg of extract protein per determination, was incubated on ice from 70 to 240 min with the indicated concentrations of [^{3}H]MAL (structure shown in *inset*). Nonspecific binding was linear with increasing [^{3}H]MAL concentrations and was 24.2 fmol (660 cpm) at 65 nM [^{3}H]MAL for the larval extract. Fitted curves were obtained using the SAS program NLIN (8), with n = 17 for each curve (see the text). Cholinesterase was 3.6 and 1.1 units/mg of protein for larvae and for adults. B. Scatchard plots of the larval and adult binding data shown in A. The fitted curves are derived from the SAS fits shown in A. ●, first stage larvae; ○, adult nematodes.

constant of 3.4 ± 0.7 nM and the adult extract (Fig. 1A, *lower curve*) consists of 1.5 ± 0.3 fmol/mg protein of saturable specific [^{3}H]MAL binding activity with a dissociation constant of 6.8 ± 1.6 nM. One fmol of [^{3}H]MAL binding corresponds to ~25 cpm. The slopes of the nonsaturating activities are 0.048 ± 0.0098 fmol/mg of protein nM^{-1} for the larval extract and 0.0084 ± 0.0098 fmol/mg of protein nM^{-1} for the adult extract. Thus, around 11 nM [^{3}H]MAL, the nonsaturating component of the larval extract is about 20% of the observed binding activity but increases to 54% at 65 nM [^{3}H]MAL, the maximum concentration at which binding activity was assayed in Fig. 1A.

Age-related changes in binding activity. The results shown in Fig. 1 demonstrate that substantial differences in binding activity exist between worm populations of different ages. These differences could be an undesirable source of variation in the comparison of binding activities present in wild-type and mutant worms. There is between 2- and 3-fold more specific [^{3}H]MAL binding activity per mg of protein in extract made from predominantly first stage larvae as compared to extract made from adult worms (Fig. 1A). Most of this decrease in specific activity with increasing age is likely to arise from a dilution effect caused by the tremendous growth of gonadal and

[1] J. A. Lewis, S. McLafferty, and J. Skimming, unpublished observation.
[2] J. A. Lewis, S. Mc Lafferty, and J. Skimming, unpublished observation.

gut tissue in maturing animals. For routine analyses, it would be impractical to collect worms of specific ages from Petri plates, as for Fig. 1. Acetylcholinesterase-specific activity is known to similarly decrease with age[3] and might provide an internal standard by which to normalize binding activity measurements made on extracts of mixed-age worms. Specific [^{3}H]MAL binding activity measured at 11 nM [^{3}H]MAL proved to be correlated with the acetylcholinesterase activity present in worms. For four different age populations of worms prepared from plates and fractionated into adult and juvenile populations by filtration through nylon mesh, the correlation coefficient between binding and esterase activities was 0.9. A correlation of 0.9 was also obtained for four mixed-age populations grown in liquid. Our results indicate that, for populations of wild-type worms grown in the same way, age-related differences in specific [^{3}H]MAL binding activity between extracts can be reasonably accounted for by normalizing binding activity to cholinesterase.

Association and dissociation rates for [^{3}H]MAL binding. The rates of association and dissociation of high affinity specific [^{3}H]MAL binding are remarkably slow for the binding of a small ligand to a receptor with an apparent K_d of about 3 nM (Fig. 2). The bimolecular rate constant for association, k_f, calculated from pseudo-first order reaction kinetics at 11 nM [^{3}H]MAL, is 1.1×10^5 M^{-1} s^{-1}. This rate is several orders of magnitude slower than expected for a diffusion-limited binding reaction and might result if a protein conformational change were required to achieve the binding state observed in our assay. The dissociation rate of [^{3}H]MAL specifically bound at an 11 nM concentration was measured after the addition of excess unlabeled MAL and occurs with a half-life of about 17 min ($k_r = 6.9 \times 10^{-4}$ s^{-1}). The calculated equilibrium dissociation constant, k_r/k_f, is about 6 nM, agreeing with the 3.4 and 6.8 nM dissociation constants predicted from equilibrium binding measurements on larval and adult extracts (Fig. 1).

Low affinity binding activity probably represents the very rapidly formed component in the forward association reaction (nonzero intercept in Fig. 2A, ~14% of the equilibrium value). Thus, it should have no effect on the determination of the forward reaction rate and, probably, being more rapidly equilibrating, would have little or no effect on the measurement of the reverse dissociation reaction rate. In attempts to measure the forward association reaction at higher [^{3}H]MAL concentrations, we found the amount of ligand immediately bound to be a much higher proportion of the final equilibrium value and much greater than predicted from the forward rate constant measured at 11 nM [^{3}H]MAL. Such binding behavior should be expected if the low affinity binding components account for the most rapidly bound ligand.

Inhibition of specific [^{3}H]MAL binding by levamisole derivatives. In Fig. 3, we show the ability of seven different levamisole derivatives to inhibit the specific [^{3}H]MAL binding measurable at 11 nM [^{3}H]MAL. The compounds tested are: unlabeled MAL; its parent compound levamisole; dexamisole, the optical and less potent isomer of levamisole; and four additional levamisole derivatives, *para*-aminolevamisole and *meta*-, *para*-, and *ortho*-nitrolevamisole. The IC_{50} concentrations of these drugs are tabulated in Table 1 and compared to the minimum concentrations of these drugs that will completely

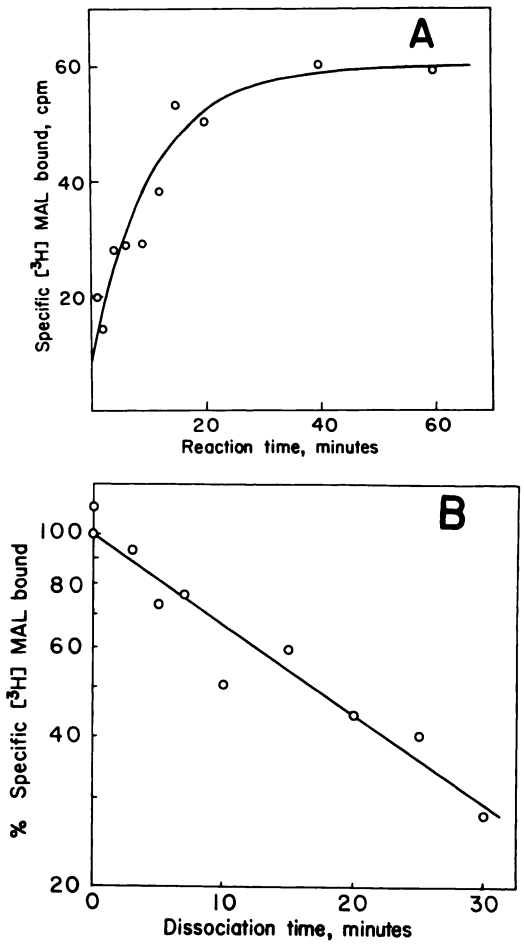

Fig. 2. Time dependence of the association and dissociation of specific [^{3}H]MAL binding. A. Association reaction. The reaction was begun by the addition of 0.74 mg of extract protein and was allowed to proceed at 11 nM [^{3}H]MAL for the indicated times on ice before dilution and filtration. B. Dissociation reaction. After incubation of 0.62 mg of extract protein for 60 min on ice with 11 nM [^{3}H]MAL, 10 μl of unlabeled MAL (0.2 mM) or distilled water were added to the respective reaction mixes for total and nonspecific binding and allowed to sit for the indicated times before dilution and filtration. Time zero points were obtained simply by dilution and filtration after the initial 60-min reaction.

contract cut nematodes within 1 hr, usually determined to the nearest 10-fold dilution. There is a good overall correspondence between the ability to inhibit specific [^{3}H]MAL binding in worm extracts and the ability to cause cut worms to contract. MAL is the most potent and *ortho*-nitrolevamisole the least potent of the derivatives tested, both *in vitro* and *in vivo*. Importantly, dexamisole, the optical isomer of levamisole, is about 10 times less potent than levamisole in either assay.

Morantel is a more potent methyl-substituted derivative of the anthelmintic pyrantel and has about the same neurotoxicity to nematodes as levamisole. Levamisole-resistant mutants are also resistant to pyrantel and morantel (1, 2). If levamisole and pyrantel compounds act in the same way, as we have predicted (2), a pyrantel derivative like morantel should powerfully inhibit specific [^{3}H]MAL binding, and we show that it does (Fig. 3, Table 1). The cholinergic effects of pyrantel compounds are much stronger on vertebrates than those of levamisole compounds and pyrantel has been accepted as a nicotine analog (17); thus, its action on the levamisole receptor buttresses the idea that the receptor might be cholinergic.

[3] R. L. Russell, personal communication.

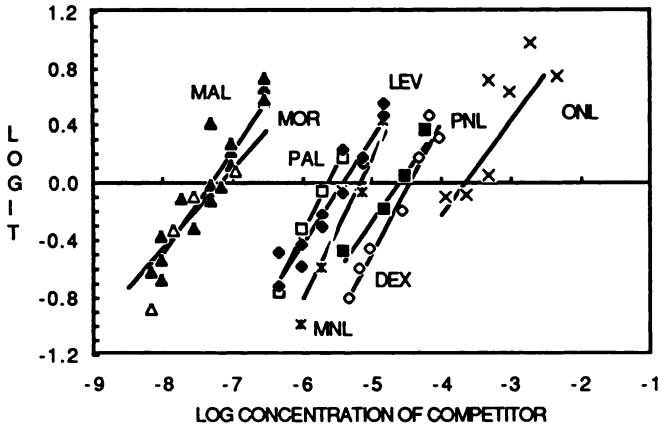

Fig. 3. Hill plots of the displacement of specific [^{3}H]MAL binding by various levamisole derivatives. Five times frozen and thawed extract was incubated for 70 min on ice with 11 nM [^{3}H]MAL and total binding was determined in the presence of the indicated drug concentrations as described in the text. Specific [^{3}H]MAL binding was calculated by subtracting nonspecific binding measured in the presence of 10 μM unlabeled MAL only, which proved the same as nonspecific binding measured with the IC_{50} concentration of each drug also present except for *ortho*-nitrolevamisole, for which nonspecific controls were done with both unlabeled MAL and the indicated concentration of the tested drug present. Values for total and nonspecific binding were typically as described in the first section of Results. If B is the amount of specific [^{3}H]MAL binding observed at a given drug concentration and B_{max} is the binding with no unlabeled drug added to the total reaction mix, then the logit is taken as $\log_{10}[(B_{max} - B)/B]$. ▲, MAL; *LEV* (●), levamisole; *DEX* (○), dexamisole; *PAL* (□), *para*-aminolevamisole; *MNL* (*dotted* ×), *meta*-nitrolevamisole; *PNL* (■), *para*-nitrolevamisole; *ONL* (×), *ortho*-nitrolevamisole; *MOR* (△), morantel.

TABLE 1

Comparison of *in vivo* and *in vitro* potencies of levamisole derivatives

The IC_{50} concentrations of various levamisole derivatives inhibiting specific [^{3}H]MAL binding are compared to the minimum concentrations of these drugs fully contracting cut *C. elegans* within 1 hr, usually determined to the nearest 10-fold concentration decrement (2). The IC_{50} concentrations were determined by weighted linear regression of the transformed data shown in Fig. 3.

Compound tested	Approximate molar concentration for	
	Threshold of response on cut worm	IC_{50} in binding assay
meta-Aminolevamisole	6×10^{-8}	4.8×10^{-8}
para-Aminolevamisole	10^{-5}	2.4×10^{-6}
Levamisole	5×10^{-7}	3.9×10^{-6}
Dexamisole	10^{-5}	3.6×10^{-5}
meta-Nitrolevamisole	10^{-5}	6.7×10^{-6}
para-Nitrolevamisole	10^{-4}	2.4×10^{-5}
ortho-Nitrolevamisole	NE (10^{-3})[a]	2.2×10^{-4}
Pyrantel derivative		
Morantel	6×10^{-7}	6.8×10^{-8}

[a] NE, not effective in causing muscle contraction at the highest concentration tested.

Inhibition of specific [^{3}H]MAL binding by cholinergic agonists. The ability of various cholinergic agonists to inhibit specific [^{3}H]MAL binding is presented in Fig. 4. The IC_{50} values observed for these agonists are summarized in Table 2 and compared to the *in vivo* potency of these compounds. Once again, a good correspondence between the *in vitro* and *in vivo* activities of these drugs is observed. The nicotinic ganglionic agonist dimethylphenylpiperazinium is the most potent cholinergic compound in both assays and the muscarinic agents muscarine and bethanecol, which are impotent in contracting

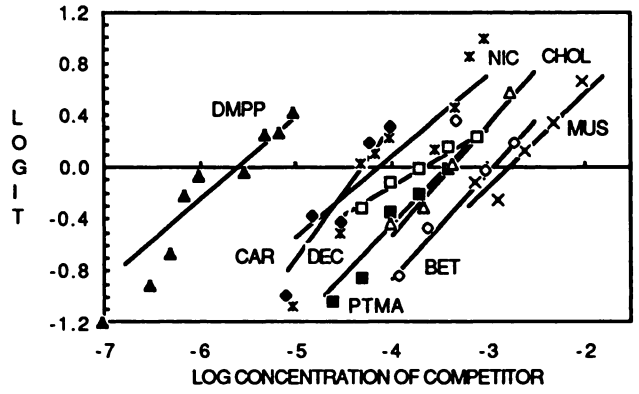

Fig. 4. Hill plots of the displacement of specific [^{3}H]MAL binding by various cholinergic agonists. As for Fig. 3, nonspecific binding was determined in the presence of 10^{-4} M unlabeled MAL and was the same as nonspecific binding measured with 10^{-4} M unlabeled MAL and the IC_{50} concentration of each drug present, except for muscarine, for which nonspecific binding controls were done with both the indicated test concentration of drug and 10^{-4} M unlabeled MAL present. *DMPP* (▲), dimethylphenylpiperazinium; *CAR* (●), carbachol; *NIC* (*dotted* ×), nicotine; *CHOL* (△), choline; *DEC* (□), decamethonium; *BET* (○), bethanecol; *PTMA* (■), phenyltrimethylammonium; *MUS* (×), muscarine.

TABLE 2

Comparison of *in vivo* and *in vitro* potencies of cholinergic agonists

The IC_{50} concentrations of various cholinergic agonists inhibiting specific [^{3}H]MAL binding are compared to the minimum concentrations of these drugs contracting cut *C. elegans* within 1 hr (2). The IC_{50} concentrations are those obtained for the data in Fig. 4, analyzed as described for Table 1.

Compound tested	Approximate molar concentration for	
	Threshold of response on cut worm	IC_{50} in binding assay
Dimethylphenylpiperazinium	3×10^{-6}	2.4×10^{-6}
Carbachol	2×10^{-5}	4.8×10^{-5}
Nicotine	10^{-4}	7.6×10^{-5}
Phenyltrimethylammonium	10^{-3}	3.8×10^{-4}
Choline	10^{-2}	4.2×10^{-4}
Decamethonium	10^{-2}, W[a]	2.5×10^{-4}
Bethanecol	NE (10^{-2})[b]	1.1×10^{-3}
Muscarine	NE (10^{-2})[b]	1.7×10^{-3}

[a] W, weak.
[b] NE, not effective in causing muscle contraction at the highest concentration tested.

cut worms, are also relatively ineffectual at inhibiting specific [^{3}H]MAL binding *in vitro*.

The overall correspondence between the *in vivo* and the *in vitro* potencies of the levamisole derivatives and cholinergic agonists shown in Tables 1 and 2 was assessed by regressing log [*in vivo* threshold concentration] against log [*in vitro* IC_{50} concentration] for those compounds in the tables effective in causing *in vivo* muscle contraction. A slope of 1.1 and a correlation coefficient of 0.90 were obtained, indicating that the *in vitro* potencies of the compounds tested are well correlated with their pharmacologically active concentrations *in vivo*.

The effects of cholinergic antagonists on specific [^{3}H]MAL binding. We have examined the effect of a number of cholinergic antagonists on specific [^{3}H]MAL binding activity. Specific [^{3}H]MAL binding at 11 nM [^{3}H]MAL was determined with the indicated concentrations of the cholinergic antagonists present in the assays for total and nonspecific binding (absence and presence of 10 μM unlabeled MAL). As Table 3 reveals, *d*-tubocurarine, atropine sulfate, quinuclidinyl benziliate, hexamethonium, and pentolinium tartrate have insignificant effects

TABLE 3

The effect of cholinergic antagonists and other compounds on specific [^{3}H]MAL binding activity

Cholinergic antagonists and several other drugs were tested for their effect on specific [^{3}H]MAL binding at the indicated drug concentrations and 11–17 nM [^{3}H]MAL concentration. Nonspecific binding was determined in the presence of 10^{-4} M unlabeled MAL with the indicated concentration of the antagonist present. The amounts of total and nonspecific binding in control extracts were typically as described in the first section of Results. The amount of specific [^{3}H]MAL binding is expressed as a percentage ratio to the amount of specific [^{3}H]MAL binding measured in a control extract with 10^{-4} M unlabeled MAL used to determine nonspecific binding. The percentage standard error was determined by the method of fractional error and thus represents a fraction of the percentage ratio figure given for each determination, not an absolute percentage relative to the control.

Compound	Concentration used	Percentage of control binding	Percentage error
	mM		
d-Tubocurarine	1	101	14
Atropine sulfate	1	91	9
Quinuclidinyl benzilate	1	131	14
Hexamethonium	1	87	14
Pentolinium tartrate	1	83	8
Mecamylamine	1	248	6
α-Bungarotoxin	0.01 mg/ml	180	6
Cobra venom	0.01 mg/ml	172	6
Eserine sulfate	20	115	11
Aldicarb	1	87	12
Procaine	1	56	11
Tricaine	1	70	11

on binding, considering the concentrations used. Since both choline and decamethonium inhibit binding (Table 2, Fig. 4), the lack of inhibition by hexamethonium was initially surprising. However, we have observed that bulky substituents close to a single trimethylammonium group generally decrease or abolish any potential inhibitory activity of a trimethylammonium group on [^{3}H]MAL binding, e.g., bethanecol versus carbachol. The impotency of hexamethonium may be attributable to the relative proximity of its two bulky trimethylammonium groups.

Mecamylamine, α-bungarotoxin, and venom of the cobra *Naja naja kaouthia* potentiate binding instead of inhibiting it. The snake toxins show some potentiating effect (10–20% activity increase) at concentrations as low as 0.001 mg/ml (~10^{-7} M). The potentiating effect is not shown by 0.1 mg/ml solutions of cytochrome *c* or poly-D,L-lysine, similarly highly cationic molecules. A significant potentiating effect (144% of control binding assayed at 11 nM [^{3}H]MAL) is obtained with mecamylamine concentrations as low as 10 μM. The optimal effect (248% of control), as shown in Table 3, is obtained at 1 mM mecamylamine. A concentration of 10 mM mecamylamine starts to inhibit [^{3}H]MAL binding (195% of control).

The acetylcholinesterase inhibitors aldicarb and eserine sulfate at 1 and 20 mM concentrations were found not to inhibit specific [^{3}H]MAL binding (Table 3). At these concentrations, they inhibit 87% and 98% of acetylcholinesterase activity. Since, conversely, levamisole is also 10–100 times more potent at inhibiting specific [^{3}H]MAL binding (Fig. 3, Table 1) than it is at inhibiting cholinesterase activity in nematode extracts (2), it does not seem likely that the cholinergic nature of [^{3}H]MAL binding activity arises from binding to a cholinesterase site.

The local anesthetics tricaine and procaine only partially inhibit [^{3}H]MAL binding at 1 mM concentrations (Table 3). The anesthetic effects these compounds have on nematodes thus are not likely to be mediated through an effect on the ligand-binding site of the levamisole receptor.

Mecamylamine and α-bungarotoxin increase receptor affinity. To further characterize the effect of mecamylamine and α-bungarotoxin, we investigated the amount of specific [^{3}H]MAL binding as a function of [^{3}H]MAL concentration in the presence and absence of 1 mM mecamylamine and, similarly, 0.01 mg/ml α-bungarotoxin. In these most recent experiments, we used more highly purified [^{3}H]MAL and we assayed a wild-type extract produced from a mixture of dauer larvae and starved juveniles, which contained 4–6 times as much receptor activity per mg of total homogenate protein as well fed worms do. Dauer larvae, a special juvenile stage formed during conditions of starvation and overcrowding (18), and the younger juveniles that survive starvation are enriched in neuronal tissue compared to non-neuronal tissue like the gut and the gonad that comprise much of the mass of older, well fed worms. With the higher activity extracts available from starved worms, we were able to show by comparing activities in the wild-type and various receptor-deficient mutants that 0.5 mM dimethylphenylpiperazinium, as an unlabeled competitor, inhibits only the high and low affinity receptor components, allowing the third, low affinity nonlevamisole receptor component to be eliminated from total specific [^{3}H]MAL binding.[4]

As shown in Fig. 5, both mecamylamine and α-bungarotoxin behave as apparent allosteric activators of specific [^{3}H]MAL binding. The effect of adding either compound was very similar. The wild-type extract in the presence of 1 mM mecamylamine assayed for 11.5 ± 1.4 fmol/mg protein of high affinity activity and 12.2 ± 1.9 fmol/mg protein of low affinity activity with dissociation constants of 1.4 ± 0.2 and 27.7 ± 14.9 nM, respectively. In the presence of 0.01 mg/ml of α-bungarotoxin, the amounts of high and low affinity binding sites were 12.5 ± 1.1

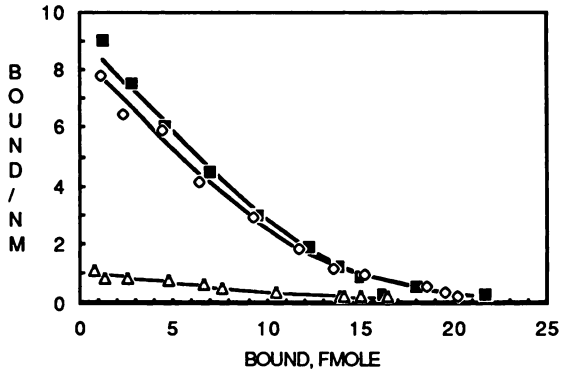

Fig. 5. Scatchard plots of specific binding of [^{3}H]MAL to nematode tissue in the presence of mecamylamine or α-bungarotoxin compared to binding in the absence of either drug. Extract from dauer larvae and starved juvenile wild-type worms, 1 mg total protein per assay, was incubated on ice for 240 min and specific [^{3}H]MAL binding was determined as described in Experimental Procedures using 0.5 mM dimethylphenylpiperazinium as the unlabeled competitor and more highly purified [^{3}H]MAL. Nonspecific binding was linear with [^{3}H]MAL concentration and was 30.2 fmol (750 cpm) at 100 nM [^{3}H]MAL, the highest concentration assayed. The fmol of specific [^{3}H]MAL bound/mg of total protein divided by the free [^{3}H]MAL ligand concentration were plotted against the specific [^{3}H]MAL bound for each mean assay point. ○, assay with 1 mM mecamylamine; ■, assay with 0.01 mg/ml α-bungarotoxin; △, assay with no additions. Fitted curves were obtained by transformation of the predicted fit to untransformed data. n = 11 for each experiment. Cholinesterase was 5.3 units/mg of protein.

[4] J. A. Lewis, S. McLafferty, and J. Skimming, unpublished results.

and 11.3 ± 2.5 fmol/mg of protein with dissociation constants of 1.4 ± 0.2 and 33.7 ± 22.1 nM, respectively. In the absence of these potentiating agents, the lower affinity activity does not saturate within the range of the assay, making it difficult to reliably determine binding parameters for both high and low affinity binding activities simultaneously using a two-hyperbola model. A hyperbola and line model can be used to accurately fit the data and determine the binding parameters of the higher affinity receptor binding component. The total binding of the high affinity site is 12.0 ± 1.1 fmol/mg of protein, with a dissociation constant of 11.7 ± 1.6 nM, with the nonsaturating activity being 0.051 ± 0.0131 fmol/mg of protein nM^{-1}. Constraining a two-hyperbola model to the binding parameters for the higher affinity site derived from the hyperbola and line model and also constraining the fit to the average total binding determined for the low affinity site in the presence of mecamylamine and α-bungarotoxin, the dissociation of the unactivated lower affinity component is estimated to be about 156 nM. The data indicate that both high and low affinity receptor components are present in about equal amounts, differ in ligand binding affinity about 20-fold, and are both activated about 5- to 8-fold by either mecamylamine or α-bungarotoxin. A curious observation is that mecamylamine consistently gave a substantially greater increase in binding activity than α-bungarotoxin or *Naja naja kaouthia* venom when the effect was measured at a single [^{3}H]MAL concentration on extracts of well fed worms using unlabeled MAL as a competitor (Table 3); the difference was not found in assaying starved worm extracts (Fig. 5).

We assayed the effect of adding mecamylamine and snake toxins simultaneously to see whether the two different agents increased specific [^{3}H]MAL binding independently of one another. Neither the further addition of α-bungarotoxin nor *Naja naja kaouthia* venom at 0.01 mg/ml caused an increase in binding beyond that caused by 1 mM mecamylamine alone. The lack of any synergistic effect between the snake toxins and mecamylamine indicates that the snake toxins and mecamylamine act in a similar manner on the same specific [^{3}H]MAL binding components.

To show that these agents do not cause the appearance of any new [^{3}H]MAL binding component not assayable in their absence, we examined the effect of mecamylamine and α-bungarotoxin on two different levamisole receptor mutants. *unc-74(x19)* is a levamisole-resistant mutant having little or no saturable specific [^{3}H]MAL binding activity measurable in the absence of mecamylamine[5] and no physiological response to levamisole (2). Fig. 6 shows that over a range of ligand concentrations there is no new specific [^{3}H]MAL binding component induced by the presence of 1 mM mecamylamine. The same mutant assayed at 17 nM [^{3}H]MAL in the presence of 0.01 mg/ml α-bungarotoxin also shows no binding activity. Similarly, the mutant *lev-1(x21) x1006rt*, which does have assayable specific [^{3}H]MAL binding activity, showed no increase in binding at 12 nM [^{3}H]MAL upon addition of either 1 mM mecamylamine, or 0.01 mg/ml α-bungarotoxin or *Naja naja kaouthia* venom. The inability of either the snake toxins or mecamylamine to potentiate binding in either type of receptor mutant shows that these compounds act on the same binding activities detected in untreated extract. The complete elimination of

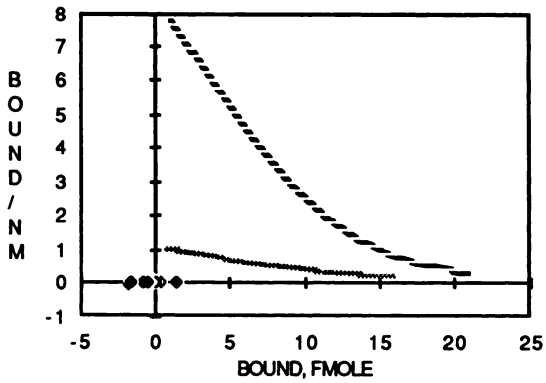

Fig. 6. Failure of mecamylamine to increase [^{3}H]MAL binding in a receptor-deficient mutant, *unc-74(x19)*. Mutant extract was assayed and the data were plotted as described for Fig. 5. ●, assay with 1 mM mecamylamine. Nonspecific binding was 25.8 fmol (640 cpm) at 100 nM [^{3}H]MAL. The theoretical fitted curves for wild-type extract assayed with and without 1 mM mecamylamine are taken from Fig. 5. Heavy broken line, wild type in the presence of mecamylamine; *light stippled line*, wild type in the absence of mecamylamine. The untransformed data were fit by a hyperbola and line model, giving 0 ± 0.44 fmol/mg protein of total saturable activity with a dissociation constant of 4.3 nM and a nondeterminable error of estimate resulting from low activity. Nonsaturable activity was 0 ± 0.007 fmol/mg of protein nM^{-1}. n = 19. Cholinesterase was 2.5 units/mg of protein.

binding activity by the *unc-74(x19)* mutation suggests that all of the binding activities detected with dimethylphenylpiperazinium as a competitor share a common genetic specification and that the complete lack of physiological response to levamisole is related to the lack of levamisole binding activity.

Discussion

Our results show that the [^{3}H]MAL binding assay we have devised detects the authentic levamisole receptor of *C. elegans*. Unlabeled MAL, the parent compound levamisole, dexamisole, and other derivatives of levamisole have the same relative potencies in inhibiting [^{3}H]MAL binding in our assay that they do in causing nematode muscle contraction (2, 19, 20), with levamisole being 10 times more potent than its stereoisomer dexamisole. The relatively slow rates observed for the formation and dissociation of bound [^{3}H]MAL also agree with studies of *Ascaris* muscle strips showing that the rates at which muscle contracture can be induced and then reversed by washing are relatively much slower for levamisole than for acetylcholine (21). The authenticity of the receptor is also corroborated by our observation of deficient or altered specific [^{3}H]MAL binding activity in mutants of all seven genes associated with extreme resistance to levamisole.[6]

The ability of cholinergic agonists to inhibit specific [^{3}H]MAL binding *in vitro* with relative potencies paralleling the *in vivo* activities of these agonists is consistent with our hypothesis (2) that the levamisole receptor is a nematode acetylcholine receptor. A more convincing demonstration of cholinergic receptor function in *C. elegans* may be achieved in the future through cytochemical localization studies and through *in vitro* reconstruction of receptor function in a situation where the influence of cholinergic agonists on ion translocation can be measured. Recently, Harrow and Gration (22) have shown that the electrophysiological responses obtained to the anthelmin-

[5] J. A. Lewis, J. T. Fleming, S. McLafferty, H. Murphy, and C. Wu, unpublished observation.

[6] J. A. Lewis, S. McLafferty, and J. Skimming, unpublished observation.

tics morantel, pyrantel, and levamisole when recording from muscle cells of the larger nematode *Ascaris* have current-voltage relationships and reversal potentials similar to those obtained with acetylcholine as an agonist. They find that acetylcholine response can be competed by coapplication of pyrantel and conclude that these anthelmintics act directly on a nematode muscle acetylcholine receptor. Future patch clamp studies on *Ascaris* may provide more detailed information that can be related to biochemical and genetic studies of the receptor found in *C. elegans*.

The dissociation constant observed for saturable specific [^{3}H]MAL binding and the IC_{50} values of the unlabeled compounds tested for inhibition of [^{3}H]MAL binding are 10- to 100-fold lower than the concentrations of these drugs required to see an immediate response in causing muscle contraction of the living worm (2). Substantial permeability barriers to the pharmacological action of these drugs probably exist in the cut worm preparation which are likely to be destroyed in preparing a homogenate for an *in vitro* receptor binding assay. Substantial occupancy of the lower affinity specific [^{3}H]MAL receptor-binding site might also be necessary to obtain a physiological response, in which case our IC_{50} values, predominantly reflecting the behavior of the higher affinity site, would systematically underestimate pharmacologically active drug concentrations.

The true *in vitro* IC_{50} values of cholinergic agonists and levamisole derivatives for inhibiting high affinity specific [^{3}H]MAL binding are probably a factor of 10 lower than the observed values shown in Tables 1 and 2. Because of limitations on assayable specific [^{3}H]MAL binding activity in well fed worm extracts, the inhibition assays were done in the presence of 11 nM [^{3}H]MAL, severalfold above the dissociation constant for the high affinity specific [^{3}H]MAL binding activity. The presence of lower affinity specific [^{3}H]MAL binding components also contributes to the need for greater concentrations of competitor to reach the point of 50% inhibition of total specific [^{3}H]MAL binding. The result is that the observed IC_{50} value for unlabeled MAL is 50 nM (Table 1), whereas the directly measured high affinity dissociation constant for [^{3}H]MAL, which has the same biological potency (10), is 5 nM. Allowing for this insensitivity in our inhibition assays, the high affinity receptor activity should then be expected to bind dimethylphenylpiperazinium with a dissociation constant of around 0.2 μM and carbachol with a dissociation constant of 5 μM. These affinities are well within the range of affinities observed for the binding of cholinergic agonists in vertebrate acetylcholine receptor assays (23).

The cholinergic antagonists tested do not inhibit specific [^{3}H]MAL binding, in contrast to the agonists. From lack of any significant *in vitro* effect on the levamisole receptor, it is likely that the weak *in vivo* blocking effects of *d*-tubocurarine and atropine (2) result from nonspecific action of these compounds. The antagonists tend to be bigger, more rigid molecules than the agonists and probably cannot fit as well as the smaller agonists into a receptor site that has undoubtedly evolved considerably from the receptor sites in vertebrates on which the antagonists have been selected to work.

We observe, though, that mecamylamine and snake neurotoxins, classical vertebrate antagonists, do have an activating effect on [^{3}H]MAL binding. Mecamylamine is known to have a strong, apparently noncompetitive physiological blocking effect on the muscle-contracting effects of levamisole compounds and cholinergic agonists (2, 24). No physiological effect has been reported for α-neurotoxins on nematodes. The physiological blocking effect of mecamylamine might be explainable if the higher affinity of [^{3}H]MAL-binding state observed *in vitro* in the presence of mecamylamine were a physiologically inactive state of the levamisole receptor. A variety of noncompetitive blocking agents are known that similarly cause the cholinergic ligand-binding site of the vertebrate nicotinic skeletal acetylcholine receptor to enter a higher affinity state [discussed by Changeux *et al.* (25)]. The lack of an observable physiological effect for snake neurotoxins might simply be due to the inability of these much larger molecules to penetrate to receptor sites *in situ*. Since the snake toxins do not inhibit [^{3}H]MAL binding as cholinergic agonists do, the activating effect the toxins do exert, if other than fortuitous, presumably occurs by interaction with some other general feature of the nematode receptor beyond the apparent cholinergic ligand-binding site.

In conclusion, we have shown that a levamisole receptor exists and possesses the pharmacological specificity expected of a receptor responsive to both the levamisole compounds and the cholinergic agonists to which the nematode is sensitive. From the developmental phenotype of levamisole-resistant mutants and from the greater resistance the mutants do show to nicotine, levamisole, and pyrantel compounds than to a close acetylcholine analog like carbachol, we expect that the levamisole receptor is but one acetylcholine receptor of the nematode and that other cholinergic receptors of differing pharmacological specificity are likely to exist. The receptor we describe, although of real significance to the animal only during the early stages of juvenile life, is useful experimentally in that it appears to be the sole determinant of sensitivity to the neurotoxic effects of levamisole. As we shall detail in further work, mutation of any one of seven genes causes alteration or deficiency of receptor binding activity, allowing a mutant animal to escape the toxic muscle-hypercontracting effects of levamisole. The ability in our system to isolate mutants affecting the function of a neuroreceptor may lead to insights on receptor regulation or expression not obtainable through a purely biochemical approach.

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