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MK-801 is a potent nematocidal agent

Characterization of MK-801 binding sites in *Caenorhabditis elegans*

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MK-801, an *N*-methyl-D-aspartate antagonist in mammalian brain tissue, is a potent nematocidal agent. Specific MK-801 binding sites have been identified and characterized in a membrane fraction prepared from the free-living nematode *Caenorhabditis elegans*. The high-affinity MK-801 binding site has an apparent dissociation constant, K_d, of 225 nM. Unlike the MK-801 binding site in mammalian tissues, the *C. elegans* binding site is not effected by glutamate or glycine, and polyamines are potent inhibitors of specific MK-801 binding.

INTRODUCTION

Glutamate binding sites may be pharmacologically distinguished by the relative affinity of selective glutamate agonists. *N*-Methyl-D-aspartate (NMDA) binding sites are activated by NMDA, whereas quisqualate and kainate are selective agonists at the non-NMDA site (Davies & Watkins, 1979; Roberts, 1981; Krogsgaard-Larsen & Honore, 1983). Stimulation of either site increases membrane permeability to univalent cations, and stimulation of NMDA receptors also produces a significant increase in calcium-ion permeability (Nowak *et al.*, 1984; MacDermott *et al.*, 1986). It has been reported that MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo-cyclohepten-5,10-imine] is a potent, non-competitive antagonist of the NMDA-type receptor (Wong *et al.*, 1986; Aram *et al.*, 1986). Electrophysiological and biochemical data suggest that MK-801 binds directly in the NMDA receptor channel complex (Wong *et al.*, 1988) and only when the channel has been opened by glutamate (Heuttner & Bean, 1988). Specific, high-affinity MK-801 binding sites have been identified and characterized in rat brain membrane preparations (Bowery & Hudson, 1986; Foster & Wong, 1987). In this paper we demonstrate that MK-801 is a potent nematocide. In addition, we describe specific, high-affinity MK-801 binding sites in *Caenorhabditis elegans*, a free-living nematode. The physiological significance of MK-801 binding sites in nematodes is unknown, although they may be involved in mediation of their nematocidal activation.

EXPERIMENTAL

Materials

[^{3}H](+)-MK-801 (37.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Purity of [^{3}H]MK-801 was confirmed to be greater than 96% using t.l.c. on silica gel with a solvent system of chloroform/methanol (9:1, v/v). (+)- and (−)-MK-801 were provided by Dr. T. Lyle and Dr. P. Anderson, Merck Sharp & Dohme Research Laboratories, West Point, PA, U.S.A. Phencyclidine was provided by the National Institute on Drug Abuse (Baltimore, MD, U.S.A.). Argiopine was synthesized as previously described (Shih *et al.*, 1988). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was purchased from Cambridge Research Biochemicals (Valley Stream, NY, U.S.A.).

Membrane preparation

C. elegans (N2 strain) was cultivated on NG agar plates covered with a lawn of *Escherichia coli* as previously described (Brenner, 1979). Worms (all stages) were washed off the plates with 5 mM-Trizma base, adjusted to pH 7.2 with HCl. The worms were washed once for 2 min at 1000 ***g***, resuspended in buffer (approx. 20000 worms/ml) and then broken up by homogenization in a Braun Homogenizer (Ace Scientific, New Brunswick, NJ, U.S.A.) using 0.5 mm glass beads for 30 s. The homogenate was centrifuged for 2 min at 1000 ***g*** and the supernatant was centrifuged for 20 min at 28000 ***g***. The resulting pellet was resuspended in buffer and washed three more times by centrifugation at 28000 ***g*** for 20 min in order to dilute cytoplasmic contaminants as much as possible. The final pellet was resuspended in Tris buffer and used immediately.

MK-801 binding

C. elegans membranes were incubated with [^{3}H]MK-801 at 22 °C for 15 min in the presence (non-specific binding) or absence (total binding) of a 500-fold molar excess of unlabelled MK-801 in glass tubes (13 mm × 100 mm). The incubation was terminated by rapid filtration over Whatman GF/B filters (presoaked for 1 h in 0.15% poly(ethylimine) in order to minimize non-specific binding), and rinsed with 15 ml (3 × 5 ml) of ice-cold Tris buffer. The filters were placed into glass vials containing 10 ml of Aquasol II (New England Nuclear), and the radioactivity was determined by liquid scintillation spec-

Abbreviations used: MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo-cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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trometry at 62 % efficiency. At saturating concentrations of [^{3}H]MK-801, the non-specific binding represented approx. 40 % of the total counts. Specific binding was determined by subtracting non-specific from total binding.

Motility assay

Worms were rinsed off the agar plates with Tris buffer at 22 °C, washed twice by centrifugation at 1000 *g* for 2 min and then resuspended in Tris buffer. Aliquots of the worms (50 μl, approx. 100 worms) were placed into 13 mm × 100 mm glass test tubes. The compounds to be tested were added to the worms in a final volume of 500 μl containing 1 % dimethyl sulphoxide. After 16 h of incubation at 22 °C, the number of worms still motile was determined by examination with a low-power dissecting microscope. More than 90 % of the worms continued to swim vigorously in the control tube.

Protein assays

Protein concentrations were determined using the dye staining technique of Bradford (1976) or in some cases following the procedure of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

C. elegans may be maintained for several days in buffer and under these conditions it is possible to evaluate the nematocidal activity of various compounds. This technique has proven to be a very successful predictor of anthelmintic activity *in vivo* (Simpken & Coles, 1981). Several putative glutamate agonists and antagonists were tested at 1 mM (Table 1), and only (+)-MK-801, (−)-MK-801, 2-amino-5-phosphonovaleric acid and glutamate diethyl ester had significant nematocidal activity. The effect of both stereoisomers of MK-801 were examined, and as shown in Fig. 1, (+)-MK-801 has an LD_{50} (concentration at which 50 % of the worms were killed) of approx. 1 μM, nearly 200-fold more potent than (−)-MK-801, demonstrating the stereospecificity of the nematocidal activity.

We developed an assay to quantify [^{3}H]MK-801 binding to membranes prepared from *C. elegans*. Specific [^{3}H]MK-801 binding increases linearly as a function of protein concentration from 11 to 104 μg of protein/ml. The optimal pH for binding was determined to be between 6.8 and 7.2, and maximal binding occurred at temperatures between 22 and 25 °C, with significantly reduced levels at temperatures less than 15 °C and greater than 42 °C. The binding of 2 μM-[^{3}H]MK-801 reached equilibrium within 5 min at 22 °C and specific binding at this concentration represents 61 % of the total binding. Specific binding sites are saturable and the Scatchard analysis of the data indicates the presence of a high-affinity site (K_d 225 nM) with a $B_{max.}$ value of 3.5 pmol/mg of protein (Fig. 2).

Pharmacological specificity of [^{3}H]MK-801 binding was evaluated by determining the amount of competition for binding sites with various related compounds (Fig. 3). The (+)-isomer of MK-801 (K_i 225 nM) is at least 50-fold more potent than the (−)-isomer (K_i 15 μM). None of the other glutamate analogues which were tested inhibited more than 10 % of the binding at concentrations up to 1.0 mM. The dissociative anaesthetics phencyclidine and (±)-ketamine are weak inhibitors of MK-801 binding to rat brain membranes (Wong *et al.*, 1986) and they do not inhibit MK-801 binding to *C. elegans* membranes at concentrations up to 1.0 mM. It was recently reported (Ransom & Stec, 1988) that selective polyamines (spermine and spermidine) stimulated MK-801 binding whereas other polyamines (cadaverine and putrescine) neither enhanced nor inhibited MK-801 binding. Interestingly, polyamines inhibit MK-801 binding to *C. elegans* membranes (Fig. 3). Four polyamines were tested (putrescine, spermidine, spermine and cadaverine) and all had similar

Table 1. Effect of glutamate analogues on *C. elegans* motility

C. elegans were maintained in the presence of various glutamate agonists, antagonists or polyamines. After 24 h, the percentage of worms still motile (as compared with a control receiving no drug) was determined. Each value is the average of three determinations; the S.E.M. for each point was less than 15 %.

Compound	% Motile
(+)-MK-801 (1 mM)	2
Glutamate diethyl ester (1 mM)	60
2-Amino-5-phosphonovaleric acid (1 mM)	65
(−)-MK-801 (1 mM)	72
Kainic acid (1 mM)	95
α-Aminoadipic acid (1 mM)	97
Glutamate (1 mM)	100
Ibotenic acid (1 mM)	100
Aspartate (1 mM)	100
N-Methyl-D-aspartate (1 mM)	100
Argiopine (30 μM)	32
Ornithine (100 μM)	100
Putrescine (100 μM)	100
Cadaverine (100 μM)	100
Spermidine (100 μM)	100
Spermine (100 μM)	100

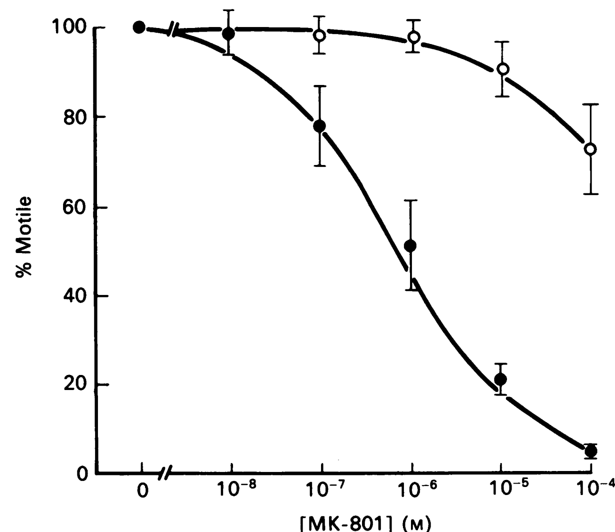

Fig. 1. MK-801 effect on *C. elegans* motility

C. elegans were maintained in the presence of increasing concentrations of either (+)-MK-801 (●) or (−)-MK-801 (○) as described in the Experimental section. After 16 h, the percentage of motile worms was determined. This experiment was replicated four times with similar results.

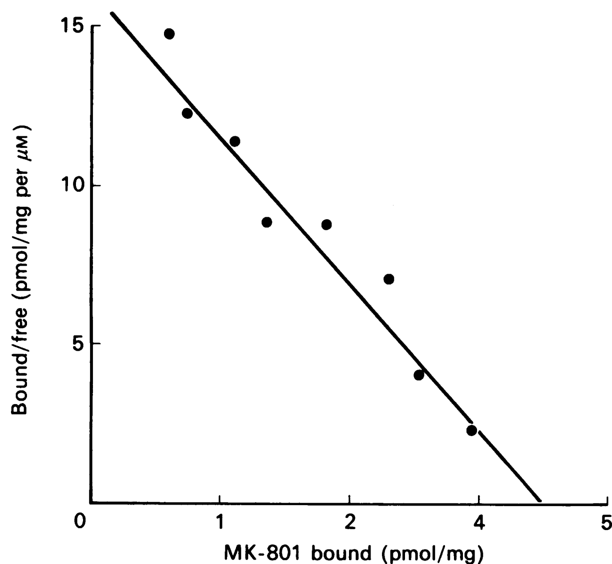

Fig. 2. Scatchard plot of [^{3}H]MK-801 binding to extensively washed *C. elegans* membranes

Each data point is the mean of three determinations; the S.E.M. was less than 15% in each case. Replicate experiments gave similar results. The K_d was 225 nM and the $B_{max.}$ was 3.5 pmol/mg.

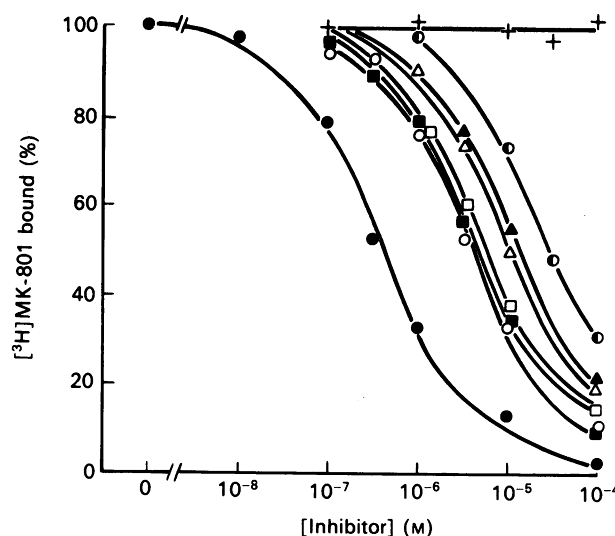

Fig. 3. Competition with specific [^{3}H]MK-801 binding to *C. elegans* membranes by putative glutamatergic agonists and antagonists

The membranes were incubated with 200 mM-[^{3}H]MK-801 in the presence or absence of various concentrations of potential inhibitors of MK-801 binding. K_i values were determined using the formula: $K_i = IC_{50}/(1 + c/K_d)$, where the IC_{50} is the concentration of the compound required to inhibit 50% of the specific binding (determined by log probit plots), and c is the concentration of [^{3}H]MK-801. The compounds examined were (+)-MK-801 (K_i = 225 nM, ●); putrescine (K_i = 2.5 μM, ○); spermine (K_i = 3 μM, ■); argiopine (K_i = 3 μM, □); cadaverine (K_i = 5 μM, △); spermidine (K_i = 9 μM, ▲); (−)-MK-801 (K_i = 15 μM, ◐) and glutamate (K_i > 1000 μM, +). Also tested were the following compounds, all of which had no effect at concentrations of 1 mM; glycine, ornithine, phencyclidine, ketamine, kainate, quisqualate, glutamate diethyl ester, ibotenic acid, 2-amino-5-phosphonovaleric acid, NMDA, AMPA, aspartate and α-aminoadipic acid.

inhibition constants (K_i values ranging from 2.5 to 9 μM; Fig. 3). The endogenous concentration of each of these compounds in *C. elegans* is not known, although the intracellular polyamine concentration in mammalian cells is in the high micromolar range (Shaw & Pateman, 1973). Ornithine (the amino acid precursor of putrescine) has no inhibitory effect at concentrations up to 1 mM. Argiopine, a polyamine-containing spider toxin reported to inhibit glutamate-stimulated sodium channels (Grishin *et al.*, 1986; Adams *et al.*, 1987), is also a potent inhibitor of MK-801 binding with a K_i value of 3 μM (Fig. 3). The significance of the polyamine effect on MK-801 binding in *C. elegans* is unknown, although in rat brain it has been suggested that there is a specific polyamine binding site on the NMDA receptor site (Ransom & Stec, 1988). Argiopine has nematocidal activity in the motility assay (32% motile at 30 μM; see Table 1); however, none of the other polyamines tested displayed nematocidal activity at concentrations up to 100 μM. The lack of bioactivity of these compounds may reflect their inability to penetrate the worm's cuticle.

Another difference between MK-801 binding sites in rat brain and *C. elegans* membranes is the effect of low concentrations of glutamate and glycine. In rat brain, MK-801 only interacts with its binding site in the open conformation, which requires the presence of an agonist such as glutamate (Foster & Wong, 1987; Heuttner & Bean, 1988). Furthermore, it has recently been reported that MK-801 binding is significantly increased in response to submicromolar levels of glycine, apparently due to a reduction in the K_d of the radioligand (Wong *et al.*, 1987; Reynolds *et al.*, 1987). In contrast, neither glutamate nor glycine has a significant effect on MK-801 binding to extensively washed *C. elegans* membranes (Fig. 3).

Bivalent cations inhibit MK-801 binding to rat brain membranes (Wong *et al.*, 1988). Their effect on MK-801 binding to *C. elegans* membranes was examined and as shown in Table 2, Ca^{2+}, Mg^{2+} and Mn^{2+} are potent inhibitors with K_i values of 30, 55 and 180 μM respectively. Univalent cations (Na^+, K^+ and Li^+) have no effect on specific MK-801 binding in *C. elegans* at concentrations up to 5.0 mM.

Table 2. Cation inhibition of [^{3}H]MK-801 binding in *C. elegans* membranes

C. elegans membranes were incubated with 200 nM-[^{3}H]MK-801 in the presence of various concentrations of the cation. The IC_{50} values (concentrations required to inhibit 50% of the specific binding) are the means of at least three determinations; the S.E.M. was less than 15% and replicate experiments gave similar results.

Cation	IC_{50} (μM)
Mg^{2+}	55
Ca^{2+}	30
Mn^{2+}	180
Na^+	> 5000
K^+	> 5000
Li^+	> 5000

In this study we have demonstrated that MK-801 is a nematocidal agent and have identified and characterized specific MK-801 binding sites in *C. elegans*. The nematocidal activity of MK-801 is stereospecific as evidenced by the 50-fold difference in potency of (+)- and (−)-MK-801 in the motility assay (Fig. 1). A similar difference in the K_i values of (+)- and (−)-MK-801 was determined (Fig. 3) which suggests that the nematocidal activity of MK-801 is mediated via a receptor-regulated mechanism. In rat brain, MK-801 interacts with the NMDA binding site and is directly involved in the regulation of calcium ion currents. *C. elegans* MK-801 binding sites do not require the presence of a glutamatergic agonist and there is no evidence that it is involved with a calcium channel.

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