The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist

(excitatory amino acid receptors/neurodegenerative diseases/phencyclidine/α opioid)

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ABSTRACT The compound MK-801 ([+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate)) is a potent anticonvulsant that is active after oral administration and whose mechanism of action is unknown. We have examined its ability to block the responses of excitatory amino acids mediated by the N-methyl-d-aspartate (N-Me-d-Asp) receptor subtype. These comprised the dissociative anesthetics phencyclidine and ketamine and the α-type opioid N-allylnormetazocine (SKF 10,047). Neurophysiological studies in vitro, using a rat cortical-slice preparation, demonstrated a potent, selective, and noncompetitive antagonistic action of MK-801 on depolarizing responses to N-Me-d-Asp but not to kainate or quissquilate. The potency of phencyclidine, ketamine, SKF 10,047, and the enantiomers of MK-801 as N-Me-d-Asp antagonists correlated closely (r = 0.99) with their potencies as inhibitors of [3H]MK-801 binding. This suggests that the MK-801 binding sites are associated with N-Me-d-Asp receptors and provides an explanation for the mechanism of action of MK-801 as an anticonvulsant.

The excitatory amino acids L-glutamate and L-aspartate are thought to act as the principal excitatory neurotransmitters in mammalian brain. The receptors mediating their actions are generally divided into the major subtypes N-methyl-D-aspartate (N-Me-D-Asp, sometimes referred to as ‘‘NMDA’’), quissqualate, and kainate, based on their activation by these selective agonists (1–5). There is considerable interest in the development of pharmacological agents that might act to block these receptors. Selective competitive antagonists of N-Me-D-Asp receptors have been developed, such as d-2-amino-5-phosphonovaleric acid and d-2-amino-7-phosphonoheptanoic acid (4, 5), but these zwitterionic compounds do not penetrate readily into the central nervous system. Nevertheless, when administered intracerebrally, N-Me-D-Asp antagonists are potent anticonvulsants (6) and have been shown to possess a remarkable ability to protect against permanent neuronal damage in animal models of cerebral ischemia and hypoglycemia (7, 8). In the present studies we describe a noncompetitive N-Me-D-Asp antagonist known from previous studies to penetrate readily into the central nervous system. The compound is MK-801 ([+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine).

MATERIALS AND METHODS

[3H]MK-801 (22.3 Ci/mmol; 1 Ci = 37 GBq) was prepared from the 7-bromo analog of MK-801 by tritium-halogen exchange. This precursor, together with samples of MK-801 and its (+) enantiomer, was kindly provided by P. Anderson (Merck Sharp & Dohme Research Laboratories, West Point, PA). Samples of (+)-N-allylnormetazocine (SKF 10,047) and phencyclidine were provided by The National Institute on Drug Abuse (Baltimore, MD). All other reagents were obtained from commercial sources.

For in vitro binding assays, cerebral cortices from male Sprague-Dawley rats (200–300 g) were homogenized in 9 volumes of ice-cold 0.32 M sucrose by nine strokes with a Teflon/glass homogenizer at 500 rpm. The homogenate was centrifuged for 10 min at 1000 × g, and the supernatant was recentrifuged at 10,000 × g for 20 min at 4°C. The pellet was suspended in assay buffer (118 mM NaCl/4.7 mM KCl/1.2 mM MgSO4/5 mM NaHCO3/20 mM Hepes/1.2 mM KH2PO4/2.5 mM CaCl2/11 mM glucose, pH 7.4) and incubated at 23°C for 20 min prior to final centrifugation at 12,000 × g for 20 min at 4°C. The pellet was resuspended in assay buffer (70 ml per gram of original tissue). Binding of [3H]MK-801 was measured by incubating 750–μl duplicate aliquots of this crude membrane suspension (≈0.75 mg of protein) with 100 μl of buffer containing displacer or of buffer alone (total

Abbreviations: N-Me-d-Asp, N-methyl-d-aspartate; aCSF, artificial cerebrospinal fluid.
binding). 100 µl of 50 nM [3H]MK-801, and 50 µl of buffer for 60 min at 23°C. Nonspecific binding was defined by 100 µM (final concentration) unlabeled MK-801.

Incubation was terminated by rapid filtration through Whatman GF/B filters, which were washed immediately with two 5-ml portions of ice-cold assay buffer in a Brandel M 24-R cell harvester. The time required for the complete filtration and washing procedure was less than 10 sec. Radioactivity on the filters was determined by liquid scintillation counting in standard vials with 10 ml of Hydrofluor (National Diagnostics, Somerville, NJ) at 41% counting efficiency. Protein concentrations were determined according to the method of Lowry et al. (12).

For in vitro neurophysiological experiments, cortical slices of rat brain were prepared in a manner similar to that described by Harrison and Simmonds (13). Coronal sections (500 µm thick) through the level of the corpus callosum were cut in artificial cerebrospinal fluid (aCSF) at room temperature, using an Oxford Vibratome. The aCSF (124 mM NaCl/2 mM MgSO4/2 mM KCl/1.25 mM KH2PO4/25 mM NaHCO3/2 mM CaCl2/10 mM glucose) was gassed continuously with an oxygen/carbon dioxide (95:5, vol/vol) mixture. Cortical wedges (=1 mm wide) were cut from these slices and mounted in a two-compartment bath, with the ventral margin of the cortical tissue traversing a greased slot which separated the chambers, so that the cortical tissue lay almost entirely within one compartment, and the white matter entirely within the other.

The chamber containing the cortical tissue (volume 0.3 ml) was continuously perfused with Mg2+-free aCSF, containing 0.1 µM tetrodotoxin to prevent spontaneous depolarizing potentials, at a rate of 1.5–2 ml/min. The dc potential between the two compartments was monitored via Ag/AgCl electrodes and a high-input impedance amplifier and continuously displayed on a chart recorder.

RESULTS

[3H]MK-801 labeled high-affinity binding sites in rat cerebral cortical membranes in a saturable manner (Fig. 2A). The binding of 5 nM [3H]MK-801 attained equilibrium after a 30-min incubation at 23°C in a near-physiological solution (assay buffer, see Materials and Methods). The “specific” binding accounted for 80% of the total binding and was abolished by pretreating the membranes at 80°C for 10 min. This binding was fully reversible in the presence of 100 µM unlabeled MK-801 (t1/2 for dissociation = 3 min) and exhibited a linear relationship with protein concentration between 0.2 and 3.2 mg per assay. Scatchard analysis of the saturation data indicated the presence of a single population of sites (Fig. 2B). Data from six experiments gave apparent affinity (Kd) and site density (Bmax) values (mean ± SEM) of 37.2 ± 2.7 nM and 0.825 ± 0.102 pmol/mg of protein, respectively.

Studies of [3H]MK-801 binding in membrane homogenates from different rat brain regions indicated a clear regional specificity (Table 1). The hippocampus exhibited the highest density of binding sites, followed by the cerebral cortex, striatum, and medulla-pons, whereas no specific binding of [3H]MK-801 was detected in membranes prepared from rat cerebellum.

Investigations of the pharmacological specificity of the [3H]MK-801 site in the cerebral cortex indicated stereoselectivity, with the (+)-isomer of MK-801 being one-seventh as potent as MK-801 [the (+)-isomer] (Fig. 3). Excitatory amino acid receptor ligands and a large number of compounds related to other neurotransmitter systems failed to show any displacement activity at concentrations up to 100 µM (Table 2). Compounds showing activity at less than 100 µM included the dissociative anesthetics phencyclidine and (±)-ketamine and the opioid receptor ligand (±)-SKF 10,047, although these were 30–250 times less potent than MK-801 itself (Fig. 3 and Table 2). Analysis of these displacement curves by a computer-assisted iterative curve-fitting program (implemented by A. Richardson, based on RSI by Bolt, Beranek and Newman, Boston, MA, 1985) indicated mass-action profiles with Hill coefficients near unity, consistent with the recognition of a single population of sites. Studies of [3H]MK-801 binding sites in hippocampus and striatum indicated that they possessed similar kinetic properties and pharmacological specificity (data not shown).

In rat cortical-slice preparations, exposure to MK-801 produced a potent blockade of depolarizing responses to N-Me-D-Asp (Figs. 4 and 5A). The threshold concentration of MK-801 for this effect was ~75 nM, which produced an

![Figure 2](https://example.com/figure2.png)

**Fig. 2.** (A) Saturation analysis of specific [3H]MK-801 binding to rat cortical membranes. Membranes were incubated with 5 nM [3H]MK-801 in the presence of increasing concentrations of unlabeled MK-801 (0.03–1.0 µM). Data are from a single experiment (which was repeated nine times, with similar results) and values are the means of triplicate determinations. (B) Scatchard plot of the specific binding data in A.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Kd, nM</th>
<th>Bmax, pmol/mg of protein</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>37.6 ± 4.4</td>
<td>1.143 ± 0.103</td>
<td>5</td>
</tr>
<tr>
<td>Cortex</td>
<td>37.2 ± 2.7</td>
<td>0.825 ± 0.102</td>
<td>6</td>
</tr>
<tr>
<td>Striatum</td>
<td>40.0 ± 3.5</td>
<td>0.654 ± 0.037</td>
<td>4</td>
</tr>
<tr>
<td>Medulla-pons</td>
<td>56.2 ± 2.9</td>
<td>0.180 ± 0.007</td>
<td>3</td>
</tr>
</tbody>
</table>

Membranes from various brain regions were incubated with [3H]MK-801 (5 nM) as described in Materials and Methods and the legend of Fig. 1. Results are mean values ± SEM of n experiments.
insurmountable blockade of N-Me-D-Asp responses (Fig. 4). This antagonism was highly selective for N-Me-D-Asp, as MK-801 in the highest concentrations tested had no effect on responses to quisqualate (30 μM MK-801) or kainate (1 μM MK-801) (Figs. 4 and 5A). The blockade of N-Me-D-Asp responses by MK-801 appeared to develop slowly, reaching a maximum only after 90–120 min of continuous superfusion with MK-801 (Fig. 4). The effect of MK-801 was persistent, with only partial recovery after a 3-h wash-out period. In Mg\(^{2+}\)-free aCSF and in the absence of tetrodotoxin, spontaneous paroxysmal depolarizing shifts were present in the majority of slices (13), and this epileptiform activity was abolished by MK-801 at concentrations that blocked N-Me-D-Asp responses.

One of the actions of dissociative anesthetics and \(\sigma\) opioids is to antagonize N-Me-D-Asp responses in a noncompetitive manner (13, 15, 16). We compared the potencies of phencyclidine, (±)-ketamine, (±)-SKF 10,047, and the enantiomers of MK-801 as antagonists of N-Me-D-Asp responses in the rat cortical slice and as inhibitors of [\(^3\)H]MK-801 binding to rat cortical membranes. With rat cortical membranes, the IC\(_{50}\) values for MK-801 and its enantiomers were 30.5 ± 1.5 nM for MK-801, 211.7 ± 25.4 nM for (−)-MK-801, 875.2 ± 37.7 nM for Phencyclidine, 4916 ± 528 nM for (±)-Ketamine, 7522 ± 721 nM for (±)-SKF 10,047, and 2580 ± 374 nM for (−)-SKF 10,047. These IC\(_{50}\) values are presented in Table 2, along with the Hill coefficients and the number of determinations (n).

Table 2. Inhibition of [\(^3\)H]MK-801 binding to rat cortical membranes

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(K_i), nM</th>
<th>Hill coefficient</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK-801</td>
<td>30.5 ± 1.5</td>
<td>0.92 ± 0.03</td>
<td>10</td>
</tr>
<tr>
<td>(−)-MK-801</td>
<td>211.7 ± 25.4</td>
<td>0.91 ± 0.04</td>
<td>5</td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>875.2 ± 37.7</td>
<td>0.89 ± 0.02</td>
<td>9</td>
</tr>
<tr>
<td>(±)-Ketamine</td>
<td>4916 ± 528</td>
<td>0.88 ± 0.01</td>
<td>5</td>
</tr>
<tr>
<td>(±)-SKF 10,047</td>
<td>7522 ± 721</td>
<td>0.99 ± 0.05</td>
<td>7</td>
</tr>
</tbody>
</table>

Membranes were incubated with [\(^3\)H]MK-801 (5 nM) as described in Materials and Methods. Results are presented as mean ± SEM of determinations. IC\(_{50}\) values and Hill coefficients were measured from data obtained using at least six concentrations of drug (in duplicates), by computer-assisted iterative curve fitting. \(K_i\) values were calculated from IC\(_{50}\) values based on the Cheng-Prusoff equation (14) using a \(K_i\) value of 38 nM for [\(^3\)H]MK-801. The following drugs gave no inhibition of [\(^3\)H]MK-801 binding when tested at concentrations up to 100 μM: L-glutamate, N-Me-D-Asp, D-aspartate, kainate, quisqualate, DL-2-amino-5-phosphonovalerate, \(\gamma\)-aminobutyrate, kojic amine, (−)-baclofen, clonazepam, picrotoxin, pentobarbital, avermectin B1a, glycine, strychnine, taurine, phenytoin, sodium valproate, ethosuximide, carbamazepine, \(\gamma\)-butyrolactone, 5-hydroxytryptamine, dopamine, haloperidol, (+)- and (−)-butacaine, norepinephrine, methamphetamine, cocaine, prazosin, phentolamine, carbachol, atropine, morphine, etorphine, naloxone, verapamil, diltiazem, 8-phenyltheophylline, ouabain, and amantadine.

**DISCUSSION**

In the present study, we used biochemical and electrophysiological techniques to identify the mechanism of action of the potent anticonvulsant MK-801 (9). The use of [\(^3\)H]MK-801 enabled the identification of high-affinity saturable binding sites with kinetic properties consistent with those of a drug receptor (Figs. 2A and 3). MK-801 binding sites have an unusual specificity; the only compounds studied that displayed any appreciable affinity for these sites were the dissociative anesthetics phencyclidine and (±)-ketamine and the \(\sigma\) opioid (±)-SKF 10,047. The close correlation between the potencies of these compounds and the enantiomers of MK-801 as antagonists of N-Me-D-Asp responses and as inhibitors of [\(^3\)H]MK-801 binding (Fig. 6) suggests that the site labeled by [\(^3\)H]MK-801 is associated with N-Me-D-Asp receptors. This site is clearly different from the N-Me-D-Asp recognition site, since neither N-Me-D-Asp itself nor any other excitatory amino acid analogs tested had any affinity for the MK-801 binding site (Table 2). However, preliminary
biochemical experiments have shown that N-Me-D-Asp receptor ligands can modulate $[^1]H$MK-801 binding properties under certain conditions, suggesting a close association between the two sites (data not shown). It has been suggested that ketamine and phencyclidine may block the ion channel operated by activation of the N-Me-D-Asp receptor (16), and thus it is possible that the MK-801 binding site may be associated with this channel. This is consistent with the noncompetitive nature of the antagonism observed with MK-801 and the similarities between the regional distribution of $[^1]H$MK-801 binding sites, N-Me-D-Asp-sensitive L-glutamate binding sites (17), and those labeled by the competitive N-Me-D-Asp antagonist d-2-amino-5-phosphono-$[^1]H$valerate (18).

The slow time course of the block of N-Me-D-Asp depolarizing responses by MK-801 was intriguing. However, we have found subsequently that the development of the antagonism does not simply relate to the length of time the tissue is exposed to MK-801 but rather to repeated additions of the agonist, N-Me-D-Asp. A possible use-dependency of the MK-801 antagonism would be in keeping with an open channel-blocking mechanism of action.

While phencyclidine, (+)-ketamine, and (+)-SKF 10,047 have modest affinities for the MK-801 site, they are also known to interact with $\sigma$-opioid receptors (19-23). Initial biochemical studies strongly suggest that the MK-801 site is not identical to the $\sigma$-opioid binding site, as MK-801 and its (−) isomer were found to possess only very low potencies in displacing the $\sigma$-opioid ligand (+)-$[^3]H$SKF 10,047 from binding sites in rat brainstem. Unlike the MK-801 site (Table