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The novel anticonvulsant MK-801 binds to the activated state of the N-methyl-D-aspartate receptor in rat brain

[1]Alan C. Foster & Erik H.F. Wong

Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

1 The influence of endogenous and exogenous acidic amino acids on the binding of [^{3}H]-MK-801, a selective, non-competitive antagonist of N-methyl-D-aspartate (NMDA) receptors, has been investigated in rat cerebral cortex crude synaptic membranes (CSM).

2 Removal of endogenous glutamate and aspartate from CSM by repeated washing reduced the affinity of [^{3}H]-MK-801 for its binding site (with no change in the total number of binding sites) and increased NMDA-sensitive L-[^{3}H]-glutamate binding.

3 In washed CSM, competitive NMDA antagonists of the DL-α-amino-ω-phosphonocarboxylate series reduced [^{3}H]-MK-801 binding and NMDA-sensitive L-[^{3}H]-glutamate binding, the most active compounds being 2-amino-5-phosphonovalerate (AP5) and 2-amino-7-phosphono-heptanoate (AP7).

4 Exogenous excitatory amino acid agonists enhanced the binding of [^{3}H]-MK-801 to washed CSM by up to 700%. A selective involvement of NMDA receptors in these effects was indicated by the excellent correlation between EC_{50}s for stimulation of [^{3}H]-MK-801 binding and IC_{50}s for inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding in the same membranes.

5 The selective, competitive NMDA receptor antagonist D-AP5 blocked the L-glutamate-induced increase in [^{3}H]-MK-801 binding in a competitive manner with a pA_2 value of 6.0.

6 These results seem to reflect a molecular interaction between two distinct components of the NMDA receptor complex: the transmitter recognition site and the site through which MK-801 exerts its antagonist effects, possibly the ion channel.

Introduction

Of the receptor subtypes which mediate the actions of the excitatory neurotransmitters L-glutamate and L-aspartate (Watkins & Evans, 1981; Foster & Fagg, 1984), the N-methyl-D-aspartate (NMDA)-preferring receptor is the best characterized, and both competitive (Evans *et al.*, 1979; 1982) and non-competitive (Lodge *et al.*, 1983; Harrison & Simmonds, 1985) NMDA receptor antagonists have been described. MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine maleate) is a novel anticonvulsant (Clineschmidt *et al.*, 1982) which acts as a potent and selective non-competitive NMDA antagonist (Wong *et al.*, 1986). Using [^{3}H]-MK-801, a unique class of high affinity binding sites can be demonstrated in rat brain membranes (Wong *et al.*, 1986; Wong & Woodruff, 1986). The non-competitive NMDA antagonists phencyclidine (PCP), ketamine and SKF 10,047 (Lodge *et al.*, 1983) compete for [^{3}H]-MK-801 binding at this site and there is an excellent correlation between their potencies as antagonists of NMDA responses in the rat cortical slice and as inhibitors of [^{3}H]-MK-801 binding (Wong *et al.*, 1986). Thus, the binding sites labelled by [^{3}H]-MK-801 appear to be the site of action of these non-competitive NMDA antagonists.

In electrophysiological experiments, the ability of MK-801 to antagonize NMDA responses is use-dependent, i.e. the degree of blockade is greatly increased by addition of NMDA receptor agonists, rather than simply being related to the length of time the tissue is exposed to MK-801 (Kemp *et al.*, 1986). In the present study, we have investigated the agonist-dependency of [^{3}H]-MK-801 binding to rat cortical membranes *in vitro* and show here that the affinity of

[1] Author for correspondence.

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[^{3}H]-MK-801 binding is dependent on agonist occupation of the NMDA receptor.

Methods

Preparation of membranes

Cerebral cortices of Sprague-Dawley (250–300 g) rats were dissected and homogenized in 9 vol (weight/vol) of ice-cold 0.32 M sucrose by 10 strokes in a glass teflon homogenizer at 500 r.p.m. All further procedures were carried out at 4°C. Homogenates were centrifuged at 1000 *g* for 10 min and the supernatant was recentrifuged at 10000 *g* for 20 min. The upper 'buffy coat' layer of the pellet was suspended in 20 vol of ice-cold water and centrifuged at 50000 *g* for 20 min. The water washing step was repeated 3 times and the resultant pellet stored at − 20°C for at least 18 h. On the day of the experiments, membrane pellets were thawed at room temperature for 30 min, suspended in 20 vol 5 mM Tris buffer (pH 7.4), centrifuged at 50000 *g* for 20 min and either used directly (denoted 'CSM') or given a further 4 cycles of washing (denoted 'washed CSM'). Each cycle consisted of resuspending the membranes in 5 mM Tris buffer (pH 7.4) and incubation at room temperature (23°C) for 20 min prior to centrifugation at 50000 *g* for 20 min. Protein content was determined by the method of Lowry *et al.* (1951).

[^{3}H]-MK-801 and L-[^{3}H]-glutamate binding assays

[^{3}H]-MK-801 binding was assayed using an incubation volume of 1 ml containing 300–500 μg membrane protein, 5 mM Tris-HCl buffer (pH 7.4), 2 nM [^{3}H]-MK-801 and test compounds. Non-specific binding was determined by the addition of 100 μM MK-801. The mixture was incubated for 45 min at 23°C and then subjected to rapid filtration through Whatman GF/B filters using a Brandell M24R cell harvester, and the filters washed by 2 × 5 ml of ice cold assay buffer (within 10 s). Filters were soaked overnight in Hydrofluor (National Diagnostics) and the radioactivity was measured by liquid scintillation counting at an efficiency of 41%.

The binding of L-[^{3}H]-glutamate was determined according to previously published methods (Fagg & Matus, 1984; Foster & Fagg, 1986) using the washed and unwashed CSM obtained above. Binding was assayed in a 50 mM Tris-acetate buffer (pH 7.0) containing 50–100 μg of membrane protein and 50 nM L-[^{3}H]-glutamate in a final volume of 0.5 ml. Non-specific binding was determined by the addition of 1 mM L-glutamate and NMDA-sensitive L-[^{3}H]-glutamate binding was defined as that displaced by 100 μM NMDA.

Determination of endogenous amino acids

CSM (before and after 5 cycles of washing) were sonicated in distilled water (6–9 mg membrane protein ml^{-1}) and centrifuged at 50000 *g* for 60 min. Glutamate and aspartate content of the supernatant was determined with an amino acid analyser (Waters 'Picotag' system).

Sources of drugs and chemicals

L-[^{3}H]-glutamate (41.5 Ci mmol^{-1}) was supplied by Dupont NEN (Boston, USA) and [^{3}H]-MK-801 (22.5 Ci mmol^{-1}) was prepared from the 7-bromo analogue of MK-801 by tritium-halogen exchange and kindly provided by Dr P. Anderson (Merck Sharp and Dohme Research Laboratories, West Point, PA, U.S.A.). L-Glutamic acid, D-aspartic acid and quinolinic acid were purchased from Sigma. The series of DL-α-amino-ω-phosphonocarboxylates together with quisqualic acid and kainic acid were obtained from Cambridge Research Biochemicals (Cambridge). N-methyl-D-aspartic acid, DL-*trans*-2,3-piperidine dicarboxylic acid, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and D-(−)-2-amino-5-phosphonovaleric acid were purchased from Tocris Chemicals (Essex). All other reagents were obtained from BDH (Dorset) and Boehringer (London).

Results

For these experiments, frozen-thawed, crude synaptic membranes (CSM) from rat cerebral cortex were used to investigate two binding sites related to the NMDA receptor. [^{3}H]-MK-801 binding sites have previously been characterized (Wong & Woodruff, 1986; Wong *et al.*, 1986) and are proposed to be the locus of action of the non-competitive NMDA antagonists such as MK-801, phencyclidine (PCP) and ketamine. L-[^{3}H]-glutamate was used to label sites in the CSM representing the transmitter recognition site of the NMDA receptor previously characterized in post-synaptic density preparations (Fagg & Matus, 1984; Foster & Fagg, 1986) and in brain sections (Monaghan *et al.*, 1985).

Effects of washing the crude synaptic membranes

The binding of [^{3}H]-MK-801 and L-[^{3}H]-glutamate was investigated in CSM before and after 5 cycles of washing with low ionic strength buffer (see Methods). [^{3}H]-MK-801 binding to CSM was characterized by a single high affinity component with a K_D of 2.24 ± 0.48 nM and a B_{max} of 1.73 ± 0.23 pmol mg^{-1} protein (n = 5; Figure 1). However, in washed CSM a

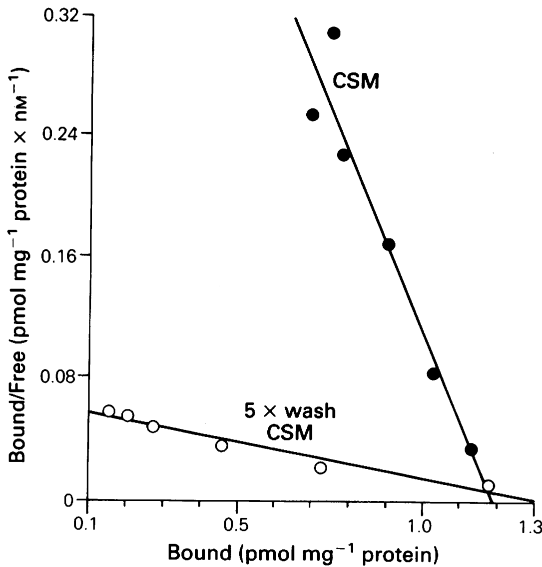

Figure 1 Influence of washing on [^{3}H]-MK-801 binding to rat cerebral cortical membranes. Scatchard analysis of [^{3}H]-MK-801 binding to CSM (●) and washed CSM (○). Assays were carried out over a range of radioligand concentrations from 0.3–1000 nM. The data are from a single experiment which was repeated 4 more times with similar results (see text for K_D and B_{max} values).

reduction of binding was apparent, due to a lowering of affinity of [^{3}H]-MK-801 for its binding site with no change of B_{max}, i.e. $K_D = 16.5 \pm 5.0$ nM, $B_{max} = 1.74 \pm 0.17$ pmol mg^{-1} protein ($n = 5$; Figure 1). In parallel experiments, the opposite effect was observed for the NMDA-sensitive binding of L-[^{3}H]-glutamate, which was increased by almost 5 fold in washed CSM (Table 1). Measurement of the content of endogenous glutamate and aspartate in the CSM indicated a 7 fold reduction in both amino acids after the washing procedure (Table 1).

Effect of a series of DL-α-amino-ω-phosphonocarboxylates on the binding of [^{3}H]-MK-801 and L-[^{3}H]-glutamate to washed crude synaptic membranes

A series of DL-α-amino-ω-phosphonocarboxylates from the 3 carbon (AP3) to the 8 carbon (AP8) members were tested as inhibitors of NMDA-sensitive L-[^{3}H]-glutamate binding and [^{3}H]-MK-801 binding to washed CSM. As has been reported previously for postsynaptic density preparations (Fagg & Matus, 1984; Foster & Fagg, 1986), maximum activity for inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding to washed CSM resides in the 5 and 7 carbon members of the series (Figure 2), indicating an NMDA receptor profile (Evans *et al.*, 1982; Olverman *et al.*, 1984). An identical pattern of lowering of binding was apparent with [^{3}H]-MK-801 (Figure 2), suggesting an interaction of this radioligand with the NMDA receptor.

Ability of excitatory amino acid agonists to enhance [^{3}H]-MK-801 binding and inhibit NMDA-sensitive L-[^{3}H]-glutamate binding to washed crude synaptic membranes

The experiments described so far indicate that [^{3}H]-MK-801 binding is increased in the presence of endogenous excitatory amino acid agonists and reduced by competitive NMDA receptor antagonists, suggesting that activation of the NMDA receptor increases the affinity of [^{3}H]-MK-801 for its binding site. This was further investigated by examining the ability of exogenous excitatory amino acid agonists to enhance the binding of [^{3}H]-MK-801 to washed CSM. As shown in Figure 3, L-glutamate, D-aspartate, NMDA, *trans*-2,3-piperidine dicarboxylate (*trans*-2,3-

Table 1 Comparison of endogenous amino acid levels and NMDA-sensitive L-[^{3}H]-glutamate binding in crude synaptic membranes (CSM) before and after washing

	CSM	*Washed CSM*	(n)
Endogenous amino acids (nmol mg^{-1} membrane protein)			
Glutamate	2.98 ± 0.31	0.42 ± 0.04	(4)
Aspartate	3.67 ± 0.33	0.54 ± 0.06	(4)
NMDA-sensitive L-[^{3}H]-glutamate binding (pmol mg^{-1} membrane protein)	0.141 ± 0.020	0.642 ± 0.047	(6)

Endogenous amino acid levels and NMDA-sensitive binding of L-[^{3}H]-glutamate were determined as described in the text using rat cortical crude synaptic membranes (CSM) before or after 5 cycles of washing. Values are the means ± s.e.mean of the number of experiments in parentheses.

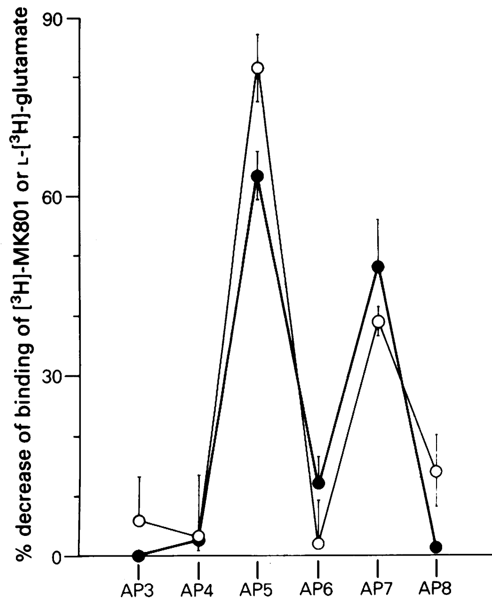

Figure 2 Reduction of [^{3}H]-MK-801 binding (●) and inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding (○) by a series of DL-α-amino-ω-phosphonocarboxylates (AP) in washed CSM. AP's were included at a concentration of 10 μM for [^{3}H]-MK-801 binding, and at 5 μM for L-[^{3}H]-glutamate binding. Values are percentage reduction of specific binding and are the means of 3–4 determinations; vertical lines show s.e.mean.

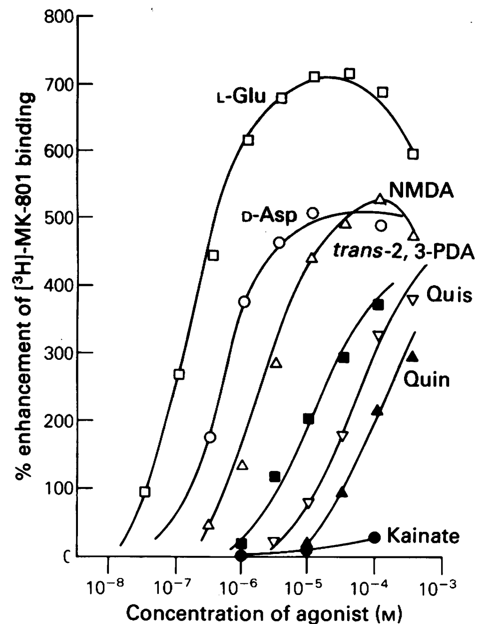

Figure 3 Enhancement of [^{3}H]-MK-801 binding to rat washed cerebral cortical membranes by excitatory amino acid agonists. Dose-response curves for L-glutamate (□), D-aspartate (○), N-methyl-D-aspartic acid (NMDA, △), *trans*-2,3-piperidine dicarboxylate (■), quisqualate (△), quinolinic acid (▲) and kainate (●). The data are from a single experiment which was repeated 3–8 times with similar results (for EC_{50} values, see Table 2). The basal value of [^{3}H]-MK-801 (2 nM final concentration) binding to washed CSM was 0.161 ± 0.018 pmol mg^{-1} protein ($n = 3$). α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) did not affect [^{3}H]-MK-801 binding at a concentration of 100 μM.

PDA), quisqualate (Quis) and quinolinic acid (Quin) each caused dose-dependent increases of [^{3}H]-MK-801 binding. At high concentrations of agonist the binding was reduced, resulting in bell-shaped curves. L-Glutamate produced a maximal enhancement of 700%, whereas that for D-aspartate and NMDA was 500%. A comparison of the EC_{50} values for stimulation of [^{3}H]-MK-801 binding and IC_{50} values for inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding in the same membranes (Table 2) gave an excellent correlation (correlation coefficient = 0.995).

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate, selective agonists for quisqualate and kainate receptors, respectively (Foster & Fagg, 1984), did not affect [^{3}H]-MK-801 binding up to 100 μM, the highest concentration tested (Figure 3).

Antagonism by D-2-amino-5-phosphonovalerate of L-glutamate-induced enhancement of [^{3}H]-MK-801 binding to washed CSM

The effect of D-AP5, a selective, competitive antagonist of NMDA receptors (Evans *et al.*, 1982), on the stimulation of [^{3}H]-MK-801 binding to washed CSM caused by L-glutamate was examined. As shown in Figure 4a, increasing concentrations of D-AP5 caused parallel, rightward shifts in the dose response curve to L-glutamate. When the data were subjected to Schild analysis (Figure 4b; Arunlakshana & Schild, 1959) a straight line was obtained with a slope of 0.98, and the pA_2 value was calculated to be 6.0.

Discussion

The results in this paper indicate that the binding of [^{3}H]-MK-801 to CSM from rat cerebral cortex is enhanced in the presence of NMDA receptor agonists of endogenous and exogenous origin, and that these

Table 2 Potencies of excitatory amino acid agonists in causing enhancement of [^{3}H]-MK-801 binding and inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding in rat washed cerebral cortical membranes

	EC_{50} (μM) against [^{3}H]-MK-801 binding	(n)	*IC_{50} (μM) against NMDA-sensitive L-[^{3}H]-glutamate binding*	(n)
L-Glutamate	0.17 ± 0.04	(8)	0.24 ± 0.08	(3)
D-Aspartate	0.47 ± 0.08	(3)	1.01 ± 0.13	(3)
NMDA	2.43 ± 0.23	(8)	4.13 ± 0.77	(3)
DL-*trans*-2,3-PDA	3.73 ± 0.48	(3)	8.04 ± 2.00	(3)
DL-Quisqualate	29.3 ± 3.9	(5)	44.5 ± 10.5	(3)
Quinolinate	193 ± 51	(4)	184 ± 58	(3)

The binding of [^{3}H]-MK-801 (final concentration = 2 nM) and L-[^{3}H]-glutamate (final concentration = 50 nM) to washed CSM was determined as described in the text. EC_{50} and IC_{50} values were calculated from dose-response curves using at least 5 concentrations of agonist. Values are the means ± s.e.mean of *n* experiments. NMDA = N-methyl-D-aspartate; DL-*trans*-2,3-PDA = DL-*trans*-2,3-piperidine dicarboxylate.

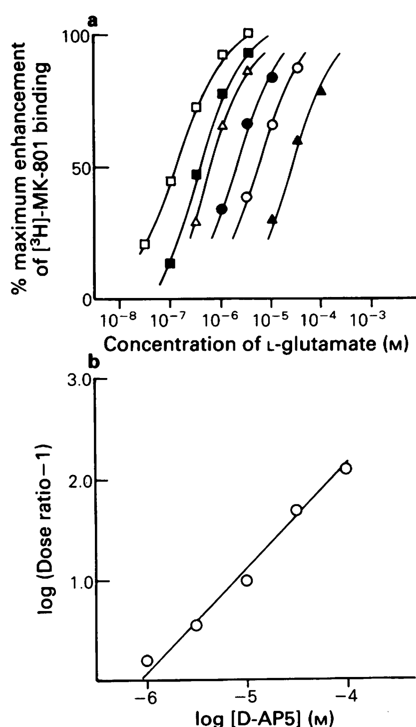

Figure 4 (a) Dose-dependent reversal of the enhancement of [^{3}H]-MK-801 binding by L-glutamate in rat cerebral cortical membranes by D-2-amino-5-phonovaleric acid (D-AP5). L-Glutamate dose-response curves were determined in the presence of 1 μM (■), 3 μM (△), 10 μM (●), 30 μM (○) and 100 μM (▲) of D-AP5. (b) Schild plot of the antagonism of L-glutamate by D-AP5 using the data from (a). Dose-ratios were calculated from EC_{50} values obtained for individual L-glutamate dose-response curves in the presence of absence of D-AP5 as determined by an iterative curve-fitting programme using Research System 1 (Bolt, Beranek and Newman, Inc.). These data are from a single experiment which was repeated with identical results.

effects are reversed by competitive NMDA receptor antagonists. The ability of endogenous glutamate and aspartate present in CSM to enhance the binding of [^{3}H]-MK-801 was evident from two observations. Firstly, repeated washing of the CSM, which caused a 7 fold reduction of endogenous glutamate and aspartate content, led to a 7 fold reduction in affinity of [^{3}H]-MK-801 for its binding site. Secondly, addition of competitive antagonists of NMDA receptors further reduced the binding of [^{3}H]-MK-801 to washed CSM. This latter effect is attributable to competition by these compounds with residual endogenous glutamate and aspartate for the transmitter recognition site of the NMDA receptor.

The fact that removal of endogenous glutamate and aspartate by washing the CSM resulted in a 5 fold enhancement of NMDA-sensitive L-[^{3}H]-glutamate binding, indicates that endogenous amino acids contaminating the membranes can occupy the transmitter recognition site of the NMDA receptor under the present binding assay conditions. Since a 2 fold increase of [^{3}H]-MK-801 binding to washed CSM was evoked by 30 nM exogenous L-glutamate (Figure 3), this suggests that the concentrations of endogenous glutamate and aspartate available to the NMDA receptor in washed CSM under binding assay conditions must be in the low nanomolar range. If all the endogenous glutamate and aspartate contaminating washed CSM was free in solution under binding assay conditions, this would give a combined concentration for those amino acids of 200–600 nM (calculated from data in Table 1). Therefore, it seems likely that the majority of endogenous glutamate and aspartate in washed CSM is not available to the receptor, perhaps remaining occluded in membrane vesicles. Nevertheless, sufficient is available to occupy the transmitter recognition sites of the NMDA receptor and cause partial enhancement of [^{3}H]-MK-801 binding in the absence of exogenous agonists.

Recently, Loo *et al.* (1986) have reported preliminary evidence that the binding of a PCP analogue [^{3}H]-1-[1-(2-thienyl)cyclohexyl]piperidine (TCP), is dependent on endogenous glutamate levels in CNS membranes, and is stimulated by exogenous glutamate. We have obtained similar results for the binding of [^{3}H]-TCP to rat brain membranes which, like [^{3}H]-MK-801, is dependent on NMDA receptor activation (unpublished observations), indicating that this phenomenon is common to these non-competitive NMDA antagonists. For [^{3}H]-MK-801, the binding appears to be almost completely dependent upon activation of NMDA receptors since in washed CSM, competitive NMDA antagonists reduce specific [^{3}H]-MK-801 binding by 90–100%.

Experiments using washed CSM indicated that the enhancement of [^{3}H]-MK-801 binding by both endogenous and exogenous excitatory amino acid agonists was due solely to activation of the NMDA receptor subtype. The reductions of [^{3}H]-MK-801 binding caused by the series of α-amino-ω-phosphonocarboxylates, maximal activity residing in DL-AP5 and DL-AP7, conform to the known profile of activity for these compounds at the NMDA receptor (Evans *et al.*, 1982; Olverman *et al.*, 1984). The excellent correlation between EC_{50} values for enhancement of [^{3}H]-MK-801 binding and IC_{50} values for inhibition of NMDA-sensitive L-[^{3}H]-glutamate binding obtained for acidic amino acid agonists is also a strong indication of an NMDA receptor-specific effect. In addition, the absolute potencies obtained are in good agreement with those previously determined for the NMDA receptor (Fagg & Matus, 1984; Olverman *et al.*, 1984; Monaghan *et al.*, 1985; Foster & Fagg, 1986). An NMDA receptor-specific effect was also apparent from the inactivity of AMPA and kainate, selective agonists for the quisqualate and kainate receptor sub-types, respectively (Watkins & Evans, 1981; Foster & Fagg, 1984). Quisqualate, an agonist which activates preferentially non-NMDA receptors (Watkins & Evans, 1981) appears to act in addition at NMDA receptors in micromolar concentrations (Olverman *et al.*, 1984; Monaghan *et al.*, 1985; Greenamyre *et al.*, 1985; Foster & Fagg, 1986). This effect has previously been ascribed to contamination of quisqualate samples by L-glutamate (Olverman *et al.*, 1984). In the present study, synthetic quisqualate was used and was found to contain no detectable L-glutamate when subjected to amino acid analysis. Thus, the ability of quisqualate to stimulate [^{3}H]-MK-801 binding with micromolar potency (Table 2) indicates an NMDA receptor-mediated effect. It is interesting to note that L-glutamate consistently produced a maximal enhancement of [^{3}H]-MK-801 binding which is greater than that for the other NMDA receptor agonists tested (Figure 3). At present, the precise reason for this difference is unclear but it may possibly be related to differing efficacies for the natural agonist, L-glutamate, and its synthetic analogues, similar to the effects of full and partial $GABA_A$-receptor agonists on benzodiazepine binding (Wong & Iversen, 1985).

Strong confirmation of NMDA receptor involvement was provided by the fact that D-AP5, a selective competitive NMDA receptor antagonist (Evans *et al.*, 1982) could inhibit the enhancement of [^{3}H]-MK-801 binding produced by L-glutamate. Increasing concentrations of D-AP5 resulted in a parallel, rightward shift in the L-glutamate dose-response curve, and Schild analysis of the data gave a slope of 1, indicating a competitive interaction between L-glutamate and D-AP5. A pA_2 value of 6 is in good agreement with previous reports of the affinity of D-AP5 at NMDA receptors (Evans *et al.*, 1982; Harrison & Simmonds, 1985; Foster *et al.*, 1986).

These biochemical data parallel the observations from electrophysiological experiments which indicate that the antagonism of NMDA-induced depolariztions in rat cortical slices by MK-801 is agonist-dependent (Kemp *et al.*, 1986). It is possible that MK-801 acts as an open channel blocker, as has been suggested for PCP and ketamine (Honey *et al.*, 1985; MacDonald & Milkjovic, 1986). Thus, the increase in [^{3}H]-MK-801 binding induced by NMDA agonists may reflect a higher affinity of this ligand for the open state of the NMDA-activated ion channel. This interaction is similar to that which occurs at the nicotinic receptor, where the binding of PCP and perhydrohistrionicotoxin to the nicotinic ion channel is enhanced when the receptor is activated by a cholinoceptor agonist (Eldefrawi *et al.*, 1980a, b). However, unlike the situation for the nicotinic receptor complex, where non-competitive antagonists increase the affinity of agonists for the acetylcholine binding site (Changeux *et al.*, 1984), we have so far been unable to detect any effect of MK-801 on the NMDA-sensitive binding of L-[^{3}H]-glutamate (unpublished observations). This also suggests the possibility that the mechanisms involved in the coupling between the transmitter recognition site and the [^{3}H]-MK-801 binding site within the NMDA receptor complex are unlike the reciprocal 'allosteric' interactions which occur within the GABA-benzodiazepine receptor complex (Olsen, 1982). At the present time it seems reasonable to conclude that the influence of NMDA agonists upon [^{3}H]-MK-801 binding represents an interaction between two closely-related components of the NMDA receptor complex, namely the transmitter recognition site and the site through which MK-801 exerts its antagonist effects, possibly the ion channel. Further investigation of these phenomena should provide insights into the molecular interactions within the NMDA receptor complex.

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