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MOLECULAR PHARMACOLOGY, 35:387-393

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Biexponential Kinetics of [^{3}H]MK-801 Binding: Evidence for Access to Closed and Open *N*-Methyl-D-aspartate Receptor Channels

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Received November 3, 1988; Accepted January 6, 1989

SUMMARY

The phencyclidine (PCP) receptor is a site within the ion channel gated by the *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor. In the present study, kinetics of association and dissociation of the specific PCP receptor ligand [^{3}H]MK-801 were determined in order to elucidate the mechanism of functioning of the NMDA receptor complex. Two distinct components of [^{3}H]MK-801 association with apparent $t_{1/2}$ values of approximately 10 min and 3 hr were resolved. Incubation with the NMDA receptor agonist L-glutamate increased the total steady state binding of [^{3}H]MK-801 and increased the relative percentage of [^{3}H]MK-801 binding that manifested fast rather than slow kinetics, without altering the observed rate constant of either the fast or slow component of association. The competitive NMDA receptor antagonist D(−)-2-amino-5-phosphonovaleric acid decreased total steady state binding of [^{3}H]MK-801. These data support a model in which [^{3}H]MK-801 can gain access to its binding site via two distinct paths, a fast hydrophilic path associated with a conformation of the NMDA receptor in which the channel is open and a slow hydrophobic path independent of the open channel. In the presence of L-glutamate, incubation with glycine increased the relative percentage of [^{3}H]MK-801 binding that manifested fast rather than slow kinetics. The Hill coefficient for stimulation of specific [^{3}H]MK-801 binding by L-glutamate was significantly greater than unity in either the absence or presence of glycine. Our data support a model of NMDA receptor functioning in which two molecules of agonist are required to convert the receptor complex to a conformation that is in equilibrium with the open conformation and in which glycine regulates the percentage of NMDA receptor complexes bound to two molecules of agonist that convert to the open configuration.

Several lines of evidence indicate that the brain receptor for the psychotomimetic dissociative anesthetic PCP is a site within the ion channel gated by the NMDA-type excitatory amino acid receptor. PCP and NMDA receptors are co-localized in the central nervous system (1–3); in addition, PCP receptor ligands have been shown to inhibit NMDA receptor-mediated conductances noncompetitively (4–13). Binding of PCP receptor ligands is enhanced by NMDA receptor agonists (e.g., L-glutamate, NMDA) (14–19) and by glycine-like amino acids (19–23) and is diminished by competitive NMDA receptor antagonists (14–19) such as D-(−)-AP5. Furthermore, PCP receptor-mediated blockade of NMDA receptor channels has been shown to be used (5–11) and voltage (8–13) dependent. These data suggest a model in which NMDA and PCP receptors represent distinct sites associated with a supramolecular NMDA receptor complex.

An important question concerns the mechanism by which PCP-like agents reach their binding site within the NMDA receptor channel. Although it has been proposed that this can occur only via open NMDA receptor channels (9), some degree of slow association of PCP-like agents has been reported even under conditions in which few if any channel openings would be expected (e.g., absence of added NMDA receptor agonists, presence of D-(−)-AP5) (7, 8, 10, 24–26). MK-801 has been shown to be a potent and selective ligand for PCP receptors (27), as well as a PCP-like noncompetitive NMDA receptor antagonist (5–7, 9–10). In the present study, kinetics of [^{3}H]MK-801 association and dissociation were determined under discrete conditions of NMDA receptor activation. Our results suggest that PCP-like agents may gain access to their binding site when channels are closed as well as open.

This work was supported in part by United States Public Health Service Grants DA-03383 to S.R.Z. and MH-00631 to D.C.J., grants from the Ritter Foundation and the David Berg Family Fund for Research in Manic-Depressive Illness to S.R.Z., and the generous support of the Department of Psychiatry of the Albert Einstein College of Medicine, Herman M. van Praag, M.D., Ph.D., Chairman.

[1] P. Ascher and J. W. Johnson, personal communication.

ABBREVIATIONS: PCP, phenycyclidine; NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid; D-(−)-AP5, D-(−)-2-amino-5-phosphonovaleric acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*, *d*]cyclohept-5,10-imine maleate; TCP, *N*-(1-[2-thienyl]cyclohexyl)piperidine; nACh, nicotinic cholinergic.

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Materials and Methods

Membrane preparation. Extensively washed crude synaptic membranes were prepared as described previously (23). Cerebral cortex plus hippocampus from male Sprague-Dawley rats (150–250 g) were rapidly removed and homogenized in 15 volumes (v/w) of ice-cold 0.32 M sucrose, using a Teflon-glass homogenizer at 800 rpm. The homogenate was centrifuged at 1000 × *g* for 10 min in a Sorvall RC-5B refrigerated centrifuge. The supernatant was decanted and recentrifuged at 20,000 × *g* for 20 min. The resulting pellet was resuspended in 30 volumes (v/w) of deionized water using a Brinkman Polytron (setting 6); the resulting suspension was centrifuged at 8000 × *g* for 20 min. The supernatant and buffy coat were decanted and centrifuged at 48,000 × *g* for 20 min. The pellet was then resuspended in 5 mM EDTA, buffered to pH 7.4 by the addition of Tris base (approximately 15 mM), and incubated for 1 hr at 37°. After incubation, the membrane suspension was again centrifuged at 48,000 × *g* for 20 min. Pellets were then frozen at −4° for 1–14 days. On the day of the experiment, frozen pellets were thawed and suspended in 30 volumes (v/w) of deionized water for 1 hr at 37°. The membrane suspension was recentrifuged for 20 min at 48,000 × *g*. Pellets were then washed three times by resuspension in 30 volumes (v/w) of deionized water and centrifugation at 48,000 × *g*. After the final centrifugation, pellets were suspended in 30–50 volumes (v/w) of 5 mM Tris-acetate buffer adjusted to pH 7.4.

Radioreceptor binding assays. For all studies, 1-ml aliquots (10–15 μg of protein) of homogenate were incubated with 1, 5, or 7.5 nM [^{3}H]MK-801 at 25° in the presence of 30 μM magnesium acetate and in the presence or absence of D-(−)-AP5, L-glutamate, or glycine as indicated. Nonspecific binding was determined in the presence of 25 μM TCP. (+)-[^{3}H]MK-801 (29.4 Ci/mmol) was obtained from Dupont/NEN (Boston, MA). D-(−)-AP5 was obtained from Cambridge Research Biochemicals (Long Beach, NY). L-Glutamate and glycine were obtained from Sigma Biochemicals (St. Louis, MO). Incubations were terminated by filtration under reduced pressure using a 24-well cell harvester (Brandel Corp., Gaithersburg, MD) and Whatman GF/B filters that had been presoaked in 0.3% polyethyleneimine. Filter disks were placed in vials containing 4 ml of Hydrofluor (National Diagnostics, Manville, NJ) and counted using an LKB 1218 Rackbeta scintillation counter at a counting efficiency of 50%. Association curves were generated by measuring specific binding of [^{3}H]MK-801 at 10–13 time points between 10 min and 48 hr. At all time points, bound [^{3}H]MK-801 represented <10% of total added ligand. For dissociation experiments, homogenate was preincubated with 1 nM [^{3}H]MK-801 overnight (16–18 hr). D-(−)-AP5, glycine, and/or L-glutamate were added immediately before initiation of the dissociation reaction by the addition of 100 μM TCP. Dissociation curves were constructed by determination of specific binding of [^{3}H]MK-801 at 10–13 time points between 5 min and 48 hr.

Data analysis. Association and dissociation experiments were analyzed using the KINETIC weighted nonlinear curve fitting program (28) implemented on an IBM-PC computer with an 80286 processor (Microsoft). For all experiments, both one- and two-exponential fits were explicitly tested. Two-exponential fits were accepted only if they were statistically superior to one-exponential fits ($p < 0.05$). Based upon the time points used, it is estimated that binding components with $t_{1/2}$ values faster than 4.5 min or slower than 160 hr would not be detectable using this technique. For determination of association (k_{+1}) and dissociation (k_{-1}) constants from association experiments, observed association constant (k_{obs}) values derived from experiments that had been conducted in the presence of 1, 5, or 7.5 nM [^{3}H]MK-801 were plotted versus [^{3}H]MK-801 concentration. k_{+1} and k_{-1} values were determined by linear regression of k_{obs} versus concentration of [^{3}H]MK-801 according to the equation:

$$k_{obs} = k_{+1} \cdot ([^3\text{H}]\text{MK-801}) + k_{-1}$$

Hill coefficients were determined by linear regression analysis of logit plots. All statistical comparisons were performed using unpaired, two-tailed, Student's *t* statistic unless otherwise specified.

Results

Kinetics of specific [^{3}H]MK-801 binding were determined by computer-assisted analysis of association curves encompassing 10–13 time points between 10 min and 48 hr (see Materials and Methods). In either the absence or presence of added L-glutamate, glycine, or D-(−)-AP5, binding reached a steady state by 24 hr with no further increase in binding occurring between 24 and 48 hr (data not shown). Under control conditions (no added L-glutamate, glycine, or D-(−)-AP5), >99% of association of 1 nM [^{3}H]MK-801 could be accounted for by a single exponential with an observed rate constant (k_{obs}) of $3.0 \pm 0.5 \times 10^{-3}$ min^{-1} (Fig. 1). In one of six experiments, a two-exponential fit of the association data was statistically superior ($p < 0.04$) to a one-exponential fit. The more rapid component displayed a k_{obs} of association of 88.3×10^{-3}min^{-1} and accounted for 4% of total steady state binding in that experiment. The variable appearance of a fast component of binding suggests that under control conditions the percentage of receptor complexes manifesting fast kinetics is below the resolution of the binding assay [approximately 10% (29)].

When association was determined in the presence of 10 μM D-(−)-AP5, binding obeyed single-exponential kinetics in all experiments. The observed rate constant of association in the presence of D-(−)-AP5 was statistically indistinguishable from the slow rate of association observed under control conditions (Table 1). Total steady state binding of 1 nM [^{3}H]MK-801 in the presence of D-(−)-AP5 was reduced to approximately 20% of control binding (Fig. 2), suggesting that residual endogenous NMDA receptor agonists remaining even in membranes that had been extensively washed (16, 17) may be responsible for allowing the binding of [^{3}H]MK-801 that was observed under control conditions.

In the presence of L-glutamate (10 μM), kinetics of 1 nM [^{3}H]MK-801 association were fit significantly better ($p < 0.05$ to p

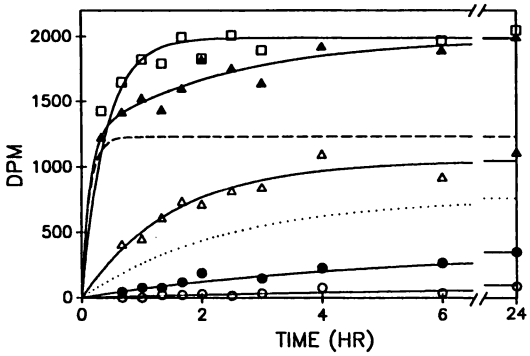

Fig. 1. Association curves of 1 nM [^{3}H]MK-801 under control conditions (●) or in the presence of 10 μM concentrations of D-(−)-AP5 (○), glycine (△), L-glutamate (▲), or L-glutamate plus glycine (□). Data shown are representative of 3–6 similar experiments. *Curves* were fitted to data points by computer-assisted nonlinear regression (see Materials and Methods). Under control conditions or in the presence of D-(−)-AP5, binding was fit best by single exponentials with apparent $t_{1/2}$ values of 3.2 and 5.8 hr, respectively. In the presence of combined L-glutamate and glycine, binding was best fit by a single exponential with an apparent $t_{1/2}$ of 12 min. In the presence of L-glutamate alone, binding was best fit by dual exponentials with apparent $t_{1/2}$ values of 5.5 min and 1.6 hr for the fast (– – –) and slow (· · · · ·) components, respectively.

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TABLE 1

Observed association constants (k_{obs}) of [3H]MK-801 binding

Values shown represent means ± standard errors of 3–5 association experiments. Statistical values were determined by one-way analysis of variance (ANOVA) unless otherwise indicated. No significant between-groups variation was found for fast or slow k_{obs} values.

	Fast Component	Slow Component
	× 10^{-3} min^{-1}	
Control	88.3[a]	3.0 ± 0.5
10 μM D-(−)-AP5	—[b]	2.3 ± 0.7
10 μM Glycine	—	6.7 ± 1.5
10 μM L-Glutamate	83.3 ± 17.8	6.4 ± 1.9
10 μM L-Glutamate + 10 μM Glycine	50.0 ± 23.3	2.7[a]

[a] Component observed in one of six experiments.
[b] Fast component of binding not detected.

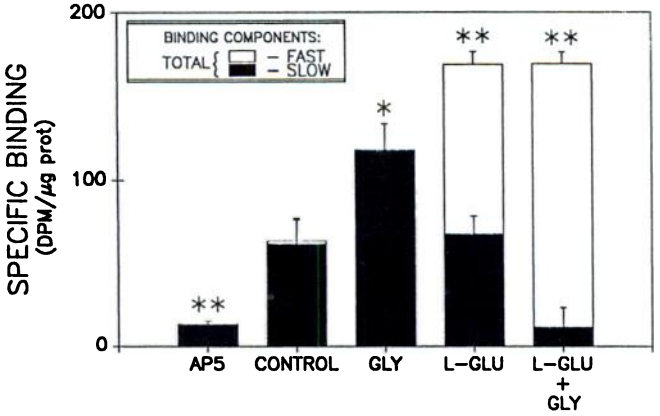

Fig. 2. Specific binding (mean ± standard error) of 1 nM [3H]MK-801 to fast (□) and slow (■) components under control conditions or in the presence of 10 μM concentrations of D-(−)-AP5 (*AP5*), glycine (*GLY*), L-glutamate (*L-GLU*), or L-glutamate plus glycine. Total bar height represents total steady state binding of [3H]MK-801 under conditions specified. Significant between-groups variation was found for fast ($p < 0.001$), slow ($p < 0.01$), and total ($p < 0.001$) steady state binding. Values represent mean ± standard error of 4–6 experiments. *$p < 0.05$ versus control. **$p < 0.01$ versus control.

< 0.0001) by a two-exponential than by a one-exponential model (Fig. 1). The observed rate constants determined in the presence of L-glutamate were statistically indistinguishable from the rate constants observed under control conditions (Table 1). In the presence of added L-glutamate, total steady state binding was significantly increased ($p < 0.01$) by 170% compared with control conditions (Fig. 2). This was due to a significant ($p < 0.001$) increase in steady state binding of [3H]MK-801 that had associated with fast kinetics, with no significant change in steady state binding of [3H]MK-801 that had associated with slow kinetics. In the presence of L-glutamate alone, 63 ± 3% of [3H]MK-801 associated with fast kinetics ($p < 0.001$ versus control).

When association of 1 nM [3H]MK-801 was determined in the presence of glycine (10 μM) alone, a single (slow) component of binding was observed. Equilibrium binding to this component was significantly increased ($p < 0.05$) by 90% relative to control conditions (Fig. 2). When incubations were conducted in the presence of combined L-glutamate and glycine, the observed rates of association for the fast and slow components were statistically indistinguishable from the rates of association observed under control conditions (Table 1). In the presence of combined L-glutamate and glycine, however, maximal binding to the fast component was significantly greater than under control conditions ($p < 0.001$) or in the presence of either L-glutamate ($p < 0.05$) or glycine ($p < 0.001$) alone (Fig. 2). The percentage of binding that associated with fast kinetics, 93 ± 7%, was increased significantly compared with both control ($p < 0.001$) and L-glutamate alone ($p < 0.05$). Total steady state binding of 1 nM [3H]MK-801 in the presence of combined L-glutamate and glycine was significantly increased by 170% over binding under control conditions ($p < 0.01$) and by 45% over binding in the presence of glycine alone ($p < 0.05$, paired two-tailed Student's t test) but was similar to binding in the presence of L-glutamate alone. Mean k_{obs} values for the fast and slow components of 1 nM [3H]MK-801 association were 66.8 ± 14.3 × 10^{-3} min^{-1} and 3.67 ± 0.55 × 10^{-3} min^{-1}, respectively.

Association of 5 and 7.5 nM [3H]MK-801 was determined under control conditions and in the presence of 10 μM concentrations of D-(−)-AP5, L-glutamate, or combined glycine and L-glutamate. Under control conditions or in the presence of D-(−)-AP5, only a single exponential of binding was observed. In the presence of L-glutamate alone or of combined glycine and L-glutamate, binding of 5 or 7.5 nM [3H]MK-801 was fit significantly better by two-exponential than by one-exponential models of association. Mean k_{obs} values for the fast and slow components of 5 nM [3H]MK-801 binding were 94.5 ± 23.8 × 10^{-3} min^{-1} and 5.17 ± 0.55 × 10^{-3} min^{-1}. Mean k_{obs} values in the presence of 7.5 nM [3H]MK-801 were 130 ± 45.3 × 10^{-3} min^{-1} and 5.20 ± 1.17 × 10^{-3} min^{-1}. In the presence of 5 nM [3H]MK-801, fast binding accounted for 65% of total in the presence of L-glutamate alone and 90% of total in the presence of combined L-glutamate and glycine. Similarly, in the presence of 7.5 nM [3H]MK-801, fast binding accounted for 53% and 85% of total in the presence of L-glutamate alone or combined L-glutamate and glycine, respectively. These values are not statistically different from the values determined for 1 nM [3H]MK-801, suggesting that MK-801 does not prevent L-glutamate- or glycine-induced conformational changes of the NMDA receptor complex.

Association (k_{+1}) and dissociation (k_{-1}) constants for the fast and slow components of [3H]MK-801 binding were determined by plotting average K_{obs} values for the fast and slow components of 1, 5, and 7.5 nM [3H]MK-801 association versus [3H]MK-801 concentration (Fig. 3; Table 2). Dissociation constants (k_{-1}) were also determined by analysis of dissociation experiments (fig. 4; Table 3). The mean value of k_1 determined by dissociation experiments for the fast component, 91.7 ± 21.6 × 10^{-3} min^{-1}, was statistically indistinguishable from the value derived from association experiments (Table 2). By contrast, the mean value of k_{-1} determined by dissociation experiments for the slow component, 0.16 ± 0.02 × 10^{-3} min^{-1}, was significantly slower than the value derived from association experiments ($p < 0.001$).

In order to clarify the mechanism by which NMDA receptor agonists influence binding of PCP receptor ligands, dose-response curves for stimulation of specific 1 nM [3H]MK-801 binding by L-glutamate were determined after 24-hr incubations in the presence or absence of 10 μM glycine. Assays were conducted in the presence of 50 μM D-(−)-AP5 in order to decrease basal binding of [3H]MK-801. In the presence of D-(−)-AP5, basal binding of [3H]MK-801 was 11.7 ± 2.5% in the absence of glycine (versus 36% under control conditions) and 17.8 ± 1.6% (versus 70% under control conditions) in the

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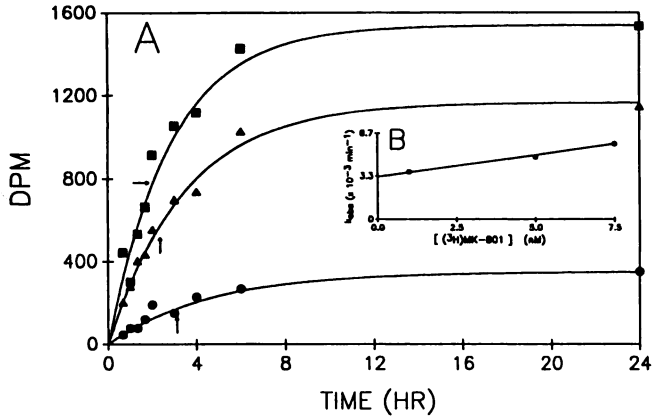

Fig. 3. Representative association curves of 1 (●), 5 (▲), and 7.5 (■) nM [^{3}H]MK-801. *Arrows* denote time points at which 50% of maximal binding is attained ($t_{1/2}$). Note progressive decrease of $t_{1/2}$ values with increasing concentration of [^{3}H]MK-801. In this experiment, which was performed under control conditions, only a single (slow) component of binding was detected. *Inset*, plot of k_{obs} versus concentration of [^{3}H]MK-801 for the single (slow) component of binding. *Line* was fit by linear regression analysis.

TABLE 2

Mean k_{+1} and k_{-1} values derived from association experiments

k_{+1} and k_{-1} values from association experiments were derived by linear regression analysis of mean k_{obs} values determined in the presence of 1 (Table 1), 5, and 7.5 nM [^{3}H]MK-801 versus [^{3}H]MK-801 concentration. k_{+1} and k_{-1} values represent the slope and *y* intercept of the linear regression lines, respectively.

	Fast Component	Slow Component
k_{+1} ($\times 10^6$ M^{-1} min^{-1})	12.3 ± 5.0	0.4 ± 0.2
k_{-1} ($\times 10^{-3}$ min^{-1})	46.7 ± 11.7	3.5 ± 0.2
K_d (nM)	3.78	8.75

presence of glycine. Glycine shifted the dose-response curve to the left (Fig. 5) but did not alter maximal [^{3}H]MK-801 binding. Hill coefficients in both the absence (1.25 ± 0.08) and presence (1.22 ± 0.06) of glycine were significantly greater than unity ($p < 0.01$ and $p < 0.05$, respectively).

Discussion

The major finding of the present study is that [^{3}H]MK-801 associates with and dissociates from its binding site in a biexponential fashion. This suggests that PCP-like agents do not gain access to their receptor only via open channels, because channel-blocking drugs that interact exclusively with open channels should manifest single-exponential association and dissociation (30). Possible explanations for the consistent detection of two distinct kinetic components of binding here but not in previous studies (20, 24–26, 31) could include 1) the use of more extensively purified membranes that might contain lower concentrations of endogenous NMDA receptor agonists and glycine-like agents, 2) the use of longer times of incubation, 3) explicit consideration of two- as well as one-exponential models of association, and 4) analysis of association in the presence of L-glutamate alone in the present study. Mg^{2+} (30 μM) has been shown to enhance [^{3}H]MK-801 binding to extensively washed membranes (31), possibly by enhancing the binding of glycine to its binding site (32). The presence of Mg^{2+}, however, cannot account for the differences between the present study and previous studies because similar concentrations of Mg^{2+} were present in some previous studies as well (24–26). The multiple kinetic components detected in the present study may account for the multiple affinity components of PCP receptor binding reported previously (18, 23).

A model of PCP-NMDA receptor interaction consistent with biexponential association of [^{3}H]MK-801 would be one in which PCP-like agents can gain access to their recognition site via two distinct paths, each corresponding to one of the observed kinetic components of binding. It has been suggested that channel-blocking drugs having pK_a values near physiological pH may associate with their binding sites via both fast hydrophilic and slow hydrophobic paths (30). At pH 7.4, MK-801 [$pK_a = 8.2$ (9)] would exist in both deprotonated and protonated forms. The former would be capable of association via both hydrophilic and hydrophobic paths whereas the latter would be capable of association only via a hydrophilic path. When channels were maximally activated in the presence of combined L-glutamate and glycine, we found that >90% of [^{3}H]MK-801 binding displayed fast kinetics of association, suggesting that the fast path represents binding of [^{3}H]MK-801 to its receptor following diffusion to the binding site via a path corresponding to the open NMDA receptor channel. In the absence of added L-glutamate or in the presence of D-(−)-AP5, >99% of [^{3}H]MK-801 binding displayed slow kinetics of association, suggesting that the slow path represents binding of [^{3}H]MK-801 following diffusion to the binding site via a path associated with closed NMDA receptors channels. The latter path could

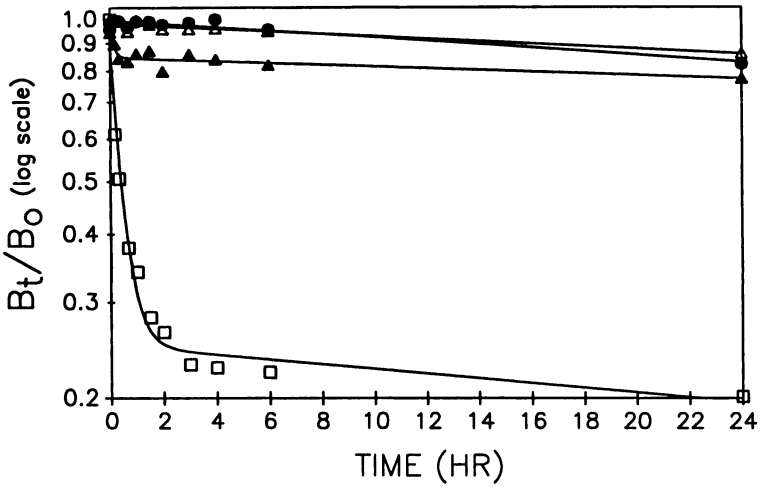

Fig. 4. Dissociation of specifically bound 1 nM [^{3}H]MK-801 from PCP receptors. Data shown are representative of 3–5 similar experiments. Membranes were preincubated for 16 hr in the presence of 1 nM [^{3}H]MK-801. Displacer (100 μM TCP) was added at *t*=0 either by itself (●) or in combination with 10 μM concentrations of glycine (△) alone, L-glutamate alone (▲), or combined L-glutamate and glycine (□). Data fit significantly better to a two- than to a one-exponential model of dissociation in the presence of either L-glutamate alone ($p < 0.04$) or combined L-glutamate and glycine ($p < 0.001$). Curves were fit to data points by computer-assisted nonlinear regression (see Materials and Methods).

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TABLE 3

k_{-1} values derived from dissociation experiments

Dissociation constants (k_{-1}) were determined based upon 3–5 dissociation experiments, each performed in triplicate. Membranes were preincubated for 16 hr with 1 nM [³H]MK-801 before initiation of displacement by addition of excess nonlabeled TCP. D-(−)-AP5, L-glutamate, and/or glycine were added along with displacer (100 μM TCP) at time zero.

	Fast Component	Slow Component
	× 10^{-3} min^{-1}	
Control	115 ± 42	0.14 ± 0.03
10 μM D-(−)-AP5	—[a]	0.21 ± 0.06
10 μM Glycine	135 ± 10	0.17 ± 0.05
10 μM L-Glutamate	118 ± 55	0.15 ± 0.06
10 μM L-Glutamate + 10 μM Glycine	48 ± 23	0.20 ± 0.05

[a] Fast component of binding not detected.

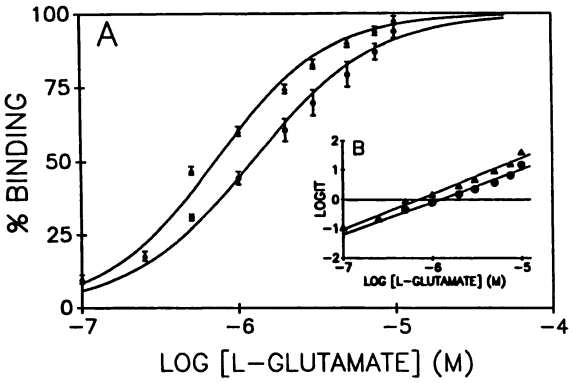

Fig. 5. Stimulation of specific 1 nM [³H]MK-801 binding by L-glutamate in the absence (●) or presence (▲) of 10 μM glycine following 24-hr incubations. Data shown represent means of 3–5 separate experiments. *Curves* were drawn based upon parameters obtained from Hill analyses. D-(M)-AP5 (50 μM) was added under all conditions to decrease basal binding. *Inset*, Hill plots of specific 1 nM [³H]MK-801 binding. Correlation coefficients (r) were >0.95 for both plots. In both the absence (●) and presence (▲) of 10 μM glycine, Hill coefficients were significantly greater than unity, supporting a four-state model of NMDA receptor functioning.

involve diffusion of deprotonated [³H]MK-801 through the lipid bilayer, through hydrophobic domains of the receptor complex, or through the (closed) NMDA receptor gating mechanism. Biexponential kinetics of association of [³H]MK-801 in the presence of L-glutamate alone indicate that association via fast and slow paths can occur simultaneously, supporting the concept that different underlying processes must be involved.

Distinct hydrophilic and hydrophobic paths underlying the fast and slow components of binding, respectively, could also account for differences between the k_{-1} value for the slow component based upon association data and that calculated from dissociation data, as observed in this study as well as reported previously (26). Results of association experiments depend only upon inward diffusion of PCP receptor ligand ([³H]MK-801). By contrast, results of dissociation studies depend upon inward diffusion of displacer (TCP) followed by outward diffusion of ligand. Under conditions in which diffusion is fast compared with binding (open channel, hydrophilic path), k_{-1} values determined from association or from dissociation studies should be identical. Conversely, when diffusion is slow compared with receptor binding (closed channel, hydrophobic path), k_{-1} values determined from dissociation studies, which depend upon diffusion of both displacer and radioligand, would be significantly lower (as we observed) than values determined by analysis of association studies.

A second finding of the present study is that L-glutamate significantly increased total [³H]MK-801 binding whereas D-(−)-AP5 significantly decreased total [³H]MK-801 binding, presumably by displacing endogenous agonists from the NMDA receptor. These data suggest that under control conditions a population of non-agonist-associated receptor complexes exists to which binding of [³H]MK-801 was not detected under our experimental conditions. Under control conditions, agonist-associated and non-agonist-associated receptors would be in equilibrium governed by the concentration of endogenous NMDA receptor agonists present. Addition of D-(−)-AP5 would displace endogenous agonists from agonist-associated receptor complexes, thus increasing the number of non-agonist-associated (nondetected) receptor complexes. Conversely, addition of L-glutamate would lead to conversion of non-agonist-associated receptor complexes to (detected) agonist-associated receptor complexes. The observation that incubation with L-glutamate alone did not decrease steady state binding to the slow component of association suggests that the slow component of association represents binding to agonist-associated, closed receptor complexes. The existence of closed, agonist-associated receptors is consistent with a recent report that high concentrations of NMDA receptor agonists fail to produce channel opening in the total absence of glycine (33).

A third finding of this study is that the Hill coefficient for stimulation of [³H]MK-801 binding by L-glutamate is significantly greater than unity. While glycine shifted the dose-response curve to the left, it did not alter the Hill coefficient significantly. This finding suggests that binding of more than one molecule of NMDA receptor agonist is required to induce channel opening. It is likely that Hill coefficients significantly greater than unity would be induced by the D-(−)-AP5 that was present in the dose-response experiments. D-(−)-AP5 is a competitive antagonist of L-glutamate at the NMDA receptor and would be expected to lead to a rightward shift in the dose-response curve for stimulation of [³H]MK-801 by L-glutamate, without affecting the steepness of the curve (34). Hill coefficients significantly greater than unity have also been observed for NMDA-induced activation of NMDA-receptor mediated conductance.[1]

Although glycine alone did not induce fast kinetics of [³H]MK-801 binding, glycine plus L-glutamate significantly increased the percentage of NMDA receptor complexes manifesting fast kinetics as compared with L-glutamate alone, suggesting that glycine activates only receptor complexes that have already bound L-glutamate. A model of NMDA receptor channel functioning consistent with our findings would be one in which there exist two agonist-associated closed conformations (RL, RL_2) in addition to open (AL_2) and agonist-dissociated closed (R) conformations (Fig. 6). Binding of two agonist molecules would be necessary to convert the NMDA receptor complex to a conformation (RL_2) from which activation (conversion to AL_2) could occur. Activation would occur by a process independent of L-glutamate concentration but regulated by glycine. Association of [³H]MK-801 with agonist-associated closed conformations (RL, RL_2) would occur with slow kinetics whereas association with the open conformation (AL_2) would occur with fast kinetics. Because association of [³H]MK-801 would occur to NMDA receptor complexes that have bound either one (RL) or two (RL_2, AL_2) molecules of agonist, Hill coefficients significantly greater than unity but less than 2 for

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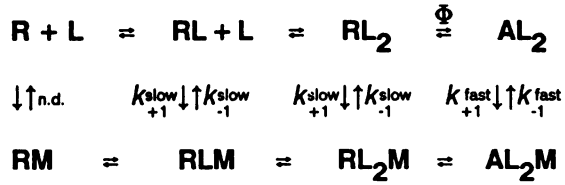

Fig. 6. Proposed model of receptor functioning (adapted in part from Refs. 37–39). *R* represents a resting (closed), agonist-dissociated conformation of the NMDA receptor complex. *RL* and RL_2 represent resting (closed), agonist-associated conformations. AL_2 represents the activated (open) conformation. *L* represents NMDA receptor agonist (i.e., L-glutamate). *M* represents [^{3}H]MK-801. Equilibria among resting conformations (*R*, *RL*, RL_2) are governed by the concentration of available L-glutamate. Equilibrium between the RL_2 (closed) and AL_2 (open) conformations is governed by a process independent of the concentration of NMDA receptor agonist and is described by the conformational equilibrium constant Φ (= $[RL_2]/[AL_2]$). Based upon the present data, binding of [^{3}H]MK-801 to agonist-associated, closed conformations (*RL*, RL_2) is postulated to manifest slow kinetics, whereas binding to the open conformation (AL_2) is postulated to manifest fast kinetics. Binding to the agonist-dissociated closed conformation (*R*) was not observed (see text). Values for Φ were calculated by dividing the density of receptor complexes that manifested fast kinetics (AL_2) by the density of complexes that manifested slow kinetics (RL_2) in the presence of a saturating concentration of L-glutamate. Densities were determined based upon equilibrium binding (Table 1) and kinetically derived K_d values (Table 2) for the fast and slow components of binding in the presence of 1, 5, and 7.5 nM [^{3}H]MK-801. The calculated value for Φ was significantly lower ($p < 0.001$) in the presence (0.24 ± 0.09) versus absence (1.25 ± 0.20) of added glycine, suggesting that glycine regulates the conformational equilibrium between closed (RL_2) and open (AL_2) conformations.

L-glutamate stimulation of [^{3}H]MK-801 binding would be expected, in agreement with our experimental data.

The proposed action for glycine would lead to a leftward shift in the dose-response curve for L-glutamate-induced stimulation of specific [^{3}H]MK-801 binding (Fig. 5) and would be consistent with studies demonstrating that glycine, when added to saturating concentrations of NMDA, increases the probability of channel opening (35, 36). The recent observation that removal of all glycine prevents NMDA-induced channel opening (33) suggests that the process mediating conversion between RL_2 and AL_2 may require the presence of glycine. The fast component of binding that we observed in the presence of L-glutamate alone, thus, might reflect the presence of endogenous glycine-like agents in our preparation. The proposed action would be consistent with the existence of a glycine-regulated gating mechanism independent of the mechanism regulated by L-glutamate.

Models analogous to that proposed here for the NMDA receptor have been suggested to account for the mechanisms of functioning of nACh (37, 38) and $GABA_A$ (39) receptors. Significant structural homology has been demonstrated between nACh and $GABA_A$ receptors, leading to the concept that they are part of a "superfamily" of ligand-gated receptor channels, which also includes strychnine-sensitive glycine receptors and may include excitatory amino acid receptors (40). The demonstration that NMDA receptors, like nACh and $GABA_A$ receptors, function according to a four-state model may, therefore, have evolutionary and structural significance. In the case of the nACh receptor, the requirement for binding of two molecules of agonist in order to induce channel opening has been shown to correspond to the existence of two agonist binding sites within each functional receptor complex (37). Noncompetitive inhibitors of the nACh receptor complex have been shown to manifest dual exponentials of association corresponding to separate hydrophilic and hydrophobic paths, the latter corresponding to diffusion through the lipid bilayer (41).

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