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ACCELERATED COMMUNICATION

Rat Brain *N*-Methyl-D-aspartate Receptors Require Multiple Molecules of Agonist for Activation

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SUMMARY

N-Methyl-D-aspartate (NMDA) receptors mediate important physiological and pathological processes, including long term potentiation and neuronal excitotoxicity. Elucidation of mechanisms underlying NMDA receptor functioning will promote understanding of the molecular bases of NMDA receptor-mediated processes. The localization of the phencyclidine (PCP) receptor within the ionophore of the NMDA receptor-gated ion channel permits the binding of PCP receptor ligands to serve as a functional marker of channel activation. We have previously demonstrated that the highly selective PCP receptor ligand [^{3}H]MK-801 displays multiexponential kinetics of association, indicating that the NMDA receptor functions according to a multistate model. Using the fast component of [^{3}H]MK-801 binding to PCP receptors as a marker for activated NMDA channels, we demonstrate here a Hill coefficient of 2 for activation of NMDA channels by L-glutamate. A multistate model of NMDA receptor functioning analogous to the model known to account for the functioning of nicotinic cholinergic and γ-aminobutyric acid$_A$ receptors fits well to our experimental data, supporting the concept that the NMDA receptor is properly classified in the Class 1 superfamily of ligand-gated channels.

NMDA receptors mediate long duration depolarizations induced by excitatory amino acids such as glutamate and aspartate. Activation of NMDA receptors is required for long term potentiation of synaptic transmission in hippocampus (1, 2) and neocortex (3) and may also contribute to excitotoxic brain damage as a consequence of hypoxia or ischemia (4, 5). A detailed understanding of the mechanisms governing NMDA receptor functioning may, thus, lead to insights into crucial physiological and pathological processes.

Quantitative studies of NMDA receptor functioning have been complicated by the multiplicity of regulatory sites associated with the NMDA receptor complex. One of these is a site at which PCP and related compounds noncompetitively antagonize NMDA receptor-mediated depolarizations by binding to a PCP receptor site within the ionophore. Because of this localization, binding of PCP receptor ligands is dependent upon the functional state of the NMDA receptor complex (6–8). Quantitative analysis of the relationship between [^{3}H]MK-801 binding and NMDA receptor activation, thus, may elucidate fundamental mechanisms governing NMDA receptor functioning. We have previously demonstrated that association of the selective high affinity ligand [^{3}H]MK-801 displays biexponential kinetics, suggesting the existence of distinct fast and slow association paths, of which the former appears to represent ligand binding to the activated (open) conformation of the NMDA receptor complex (9). For the present study, the effect of L-glutamate upon each kinetic component of [^{3}H]MK-801 binding was quantified in order to determine the agonist requirement for NMDA receptor activation and to progress towards development of a model of NMDA receptor functioning.

Materials and Methods

Membrane preparation. Extensively washed crude synaptic membranes were prepared as described previously (10). Cerebral cortex plus hippocampus from male Sprague-Dawley rats (150–250 g) were rapidly removed and homogenized in 15 ml of ice-cold 0.32 M sucrose/g, using a Teflon-glass homogenizer at 800 rpm. The homogenate was centrifuged at 1,000 × *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 15 ml of deionized water/g, using a Brinkman Polytron (setting 6); the resulting suspension was centrifuged at 8,000 × *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, which was buffered to pH 7.4 by the

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; D-(−)-AP5, D-(−)-2-amino-5-phosphonovaleric acid; GABA, γ-aminobutyric acid.

addition of Tris base (approximately 15 mM), and incubated for 1 hr at 37°. Following 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 experiment, frozen pellets were thawed and suspended in 15 ml of deionized water/g, 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 15 ml of deionized water/g and centrifugation at 48,000 × g. Following the final centrifugation, pellets were suspended in 30–50 ml of 5 mM Tris-acetate buffer/g, 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 nM [^{3}H]MK-801 at 25°, in the presence of 30 μM magnesium acetate, 50 μM D-(−)-AP5, 100 μM glycine, and varying concentrations of L-glutamate as indicated. Nonspecific binding was determined in the presence of 10 μM MK-801. (+)-[^{3}H]MK-801 (29.0 and 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 12–14 time points between 10 min and 24 hr. We have previously demonstrated that no significant further association occurs between 24 and 48 hr (9). At all time points, bound [^{3}H]MK-801 represented <10% of total added ligand.

Data analysis. Association experiments were analyzed using the KINETIC weighted nonlinear curve-fitting program, implemented on an IBM-PC computer. 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 ($p < 0.05$).

Hill coefficients for stimulation of fast binding by L-glutamate were determined using linear regression analysis of log-logit plots, according to the equation

$$\log (R_{eq}/(R_{max} - R_{eq})) = n \log ([\text{glutamate}]) - \log K_{eff}$$

where R_{eq} represents steady state binding to the fast component at a given concentration of L-glutamate, R_{max} represents steady state binding to the fast component in the presence of a saturating concentration of L-glutamate (100 μM), K_{eff} represents the effective K_d for L-glutamate binding, and n represents the Hill coefficient.

Computer simulations were performed using the Quattro spreadsheet, implemented on an IBM-PC computer. Correlations between predicted and observed values were determined using linear regression analyses, with y-intercepts constrained to zero. Slopes for all correlation curves were statistically indistinguishable from unity. For statistical comparisons, correlation coefficients were converted to z scores according to the formula $z = 0.5 \cdot \ln((1 + r)/(1 - r))$. All statistical comparisons were performed using unpaired, two-tailed, Student's t statistic, unless otherwise specified.

Results

Specific binding of 1 nM [^{3}H]MK-801 to extensively washed, frozen-thawed membranes derived from rat forebrain and hippocampus was measured at 12–14 time points between 10 min and 24 hr. We have previously demonstrated that no further association of [^{3}H]MK-801 occurs between 24 and 48 hr, even in the presence of D-(−)-AP5 (9). Association kinetics were determined using the KINETIC nonlinear curve-fitting program (11). Studies were conducted in the presence of D-(−)-AP5 (50 μM), which was added to control for the presence of residual endogenous NMDA receptor agonists. Glycine (100 μM), which potentiates (12) and may serve as a necessary cofactor for (13) NMDA receptor activation, and Mg^{2+}, which in micromolar concentrations potentiates PCP receptor binding (6), were also present under all conditions, in order to optimize [^{3}H]-MK-801 binding and response to agonist. The concentration of Mg^{2+} used (30 μM) was much lower than the concentration that inhibits [^{3}H]MK-801 binding (10 mM) (14).

Association experiments were performed in the absence and presence of added L-glutamate (0.3–100 μM) (Fig. 1). Under all conditions, biexponential models of association proved to be statistically superior ($p < 0.05$ to $p < 0.001$) to single-exponential models. The mean observed rate constants for the fast and slow components were 0.14 ± 0.04 and 0.0089 ± 0.0011 min^{-1}, respectively. At all time points, total [^{3}H]MK-801 binding represented the sum of these distinct fast and slow components.

Under control conditions, a low level of [^{3}H]MK-801 binding could be detected, which may reflect a small degree of partial agonist activity of D-(−)-AP5. This possibility is consistent with the observations that D-(−)-AP5 inhibition curves are significantly more shallow in the absence as compared with the presence of L-glutamate (6), that the IC_{50} for inhibition of PCP receptor binding by D-(−)-AP5 is significantly higher than the IC_{50} for inhibition of L-[^{3}H]glutamate binding (8), and that extremely high doses of DL-AP5 (1 mM) may have an agonist-like effect on [^{3}H]MK-801 dissociation (14). The presence of residual excitatory amino acids even in well washed, extensively treated membranes (15) may also contribute to the low level of control binding. Incubation in the presence of L-glutamate led to a significant ($p < 0.001$) increase in total steady state [^{3}H]-MK-801 binding, to a maximum of 450% of control (Fig. 2). The increase in total binding was reflected in significant increases in computer-determined steady state binding to both the fast and slow components of association.

In the presence of L-glutamate, steady state binding of [^{3}H]-MK-801 displaying fast kinetics of association increased significantly, as determined by analysis of variance ($F_{6,32} = 24.8$,

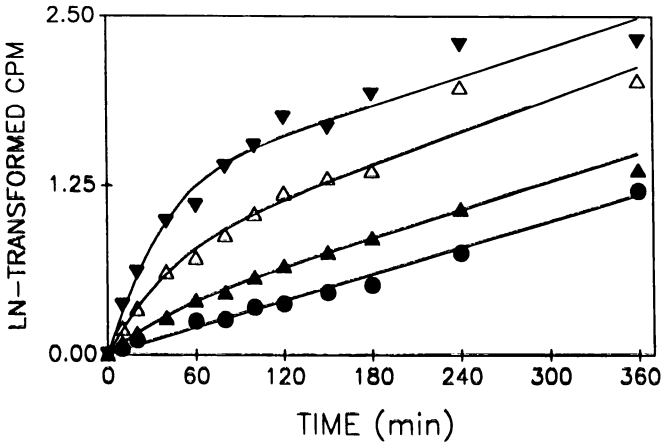

Fig. 1. Representative curves for association of 1 nM [^{3}H]MK-801 to PCP receptors under control conditions (●) and in the presence of 1 (▲), 3 (△), and 10 (▼) μM L-glutamate. Raw data (cpm) have been transformed according to the equation (ln-transformed cpm) = $\ln(cpm_{eq}/(cpm_{eq} - cpm_t))$, where cpm_{eq} represents steady state binding of 1 nM [^{3}H]MK-801 (determined following 24-hr incubation) and cpm_t represents binding at time points indicated on the *abscissa*. Data transformed in this manner yield a straight line if association is adequately fit by a single exponential function and are curvilinear if association is multiexponential.

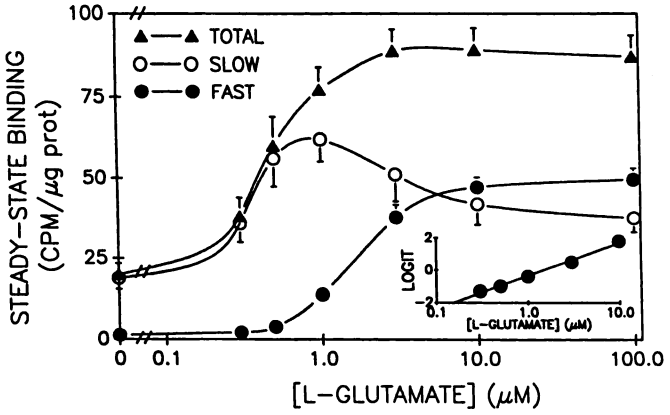

Fig. 2. Dose-response curves for L-glutamate-induced stimulation of steady state binding of [^{3}H]MK-801, as determined by analysis of association curves (Fig. 1). *Points*, means ± standard errors of six separate experiments. *Error bars* not shown are within *symbols*. *Inset*, Hill plot of dose-response data for stimulation of steady state binding of rapidly associating [^{3}H]MK-801 by L-glutamate. *Line*, drawn by linear regression ($r > 0.98$).

$p < 0.0001$), in a dose-dependent fashion. This increase was not accompanied by a significant alteration in the observed rate of fast [^{3}H]MK-801 association ($F_{6,32} = 1.55$, not significant). The Hill coefficient for stimulation of fast [^{3}H]MK-801 binding by L-glutamate, 2.06 ± 0.08, was significantly greater than unity. By contrast, the addition of L-glutamate induced a biphasic effect on steady state binding of [^{3}H]MK-801 that displayed slow kinetics of association. Concentrations of L-glutamate up to 1 μM led to a significant ($p < 0.001$) dose-dependent increase in slow [^{3}H]MK-801 binding, to a maximum of 330% of the control level. Concentrations of L-glutamate greater than 1 μM induced a subsequent dose-dependent decline in slow [^{3}H]MK-801 binding, to 210% of control. However, slow [^{3}H]MK-801 binding remained significantly higher ($p < 0.05$) than under control conditions even in the presence of a saturating concentration of L-glutamate (100 μM). The percentage of steady state [^{3}H]MK-801 binding associating with fast kinetics increased from 5.7 ± 1.7 to a maximum of 57.3 ± 3.0% ($p < 0.001$ over control) as a function of L-glutamate concentration.

Discussion

The present study provides the first demonstration that stimulation of fast binding of [^{3}H]MK-801 by L-glutamate manifests a Hill coefficient significantly greater than unity. The $t_{1/2}$ of fast association observed in the present study, 4.2 min, is similar to the $t_{1/2}$ of 1.8 min that would be expected based upon kinetic measurements of MK-801-induced open channel blockade in intact neurons (16), supporting our previous suggestion that fast [^{3}H]MK-801 binding corresponds to association via the open NMDA channel. The observation that dissociation from closed agonist-associated complexes occurs at 0.004–0.08 times the rate of dissociation from open complexes (9) suggests that complexes that manifest fast association are those whose mean open time, on average, is greater than 0.4–8.0% of total. Based upon patch-clamp studies of single NMDA channels, it has been concluded that, under conditions of maximal NMDA receptor activation, NMDA channels remain in the open conformation approximately 0.2–0.7% of the time (16).

We have previously proposed that steady state binding to the fast component may provide a measure of the percentage of complexes that would be considered maximally activated in electrophysiological studies. The present study provides the first demonstration that fast [^{3}H]MK-801 binding can be treated as a continuous variable that shows a classical sigmoidal dependence upon agonist (L-glutamate) concentration. The Hill coefficient of 2 that we observed for stimulation of fast [^{3}H]-MK-801 binding by L-glutamate suggests that binding of at least two molecules of L-glutamate is required for NMDA receptor activation.

The present data are consistent with electrophysiological studies performed using rat cortical slices (17) and cultured neurons (18), which have demonstrated Hill coefficients significantly greater than unity for activation of NMDA receptors by L-glutamate. By contrast, studies performed on NMDA receptors expressed in *Xenopus* oocytes have been interpreted as being consistent with a Hill coefficient of unity (19, 20). It has recently been demonstrated that $GABA_A$ receptors expressed in *Xenopus* oocytes manifest a Hill coefficient of unity for activation, as compared with native receptors, which manifest a Hill coefficient of 2 (21). The differential agonist requirement has been postulated to result from incomplete assembly of the receptor complex in *Xenopus* oocytes. The present study, which was performed using tissue derived from rat brain, suggests that at least two molecules of L-glutamate are required for activation of the native NMDA receptor complex.

We have previously proposed (9, 10) that NMDA receptor activation may be accounted for by the reaction scheme,

$$R + L \leftrightarrow RL + L \leftrightarrow RL_2 \leftrightarrow AL_2 \qquad (1)$$

in which R, RL, and RL_2 represent resting (nonactivated) conformations of the NMDA receptor complex (R) associated with 0, 1, and 2 molecules of L-glutamate (L), respectively. AL_2

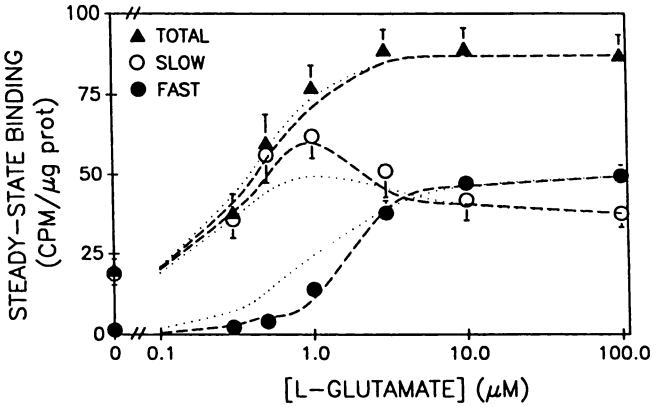

Fig. 3. Comparison of predicted and observed values for total steady state binding and for steady state binding of rapidly and slowly associating [^{3}H]MK-801. · · · · ·, Predicted values based upon the assumption that the conformational equilibrium constant (Φ) is independent of L-glutamate concentration. – – –, Predicted values in which the conformational equilibrium constant is postulated to decrease in value as a function of L-glutamate concentration. Values of Φ at each concentration of L-glutamate were calculated by dividing the percentage of slow binding that was calculated to correspond to binding to the RL_2 component, according to Eq. 2 and 3, by the value for steady state binding to the fast component (AL_2). The mean value of Φ for the three lowest concentrations (0.3–1.0 μM) was 2.38 ± 0.61, whereas the mean value for the three highest concentrations (3.0–100 μM) was 0.79 ± 0.04. These values, thus, correspond to probabilities of channel opening subsequent to L-glutamate binding of 31 and 56%, respectively.

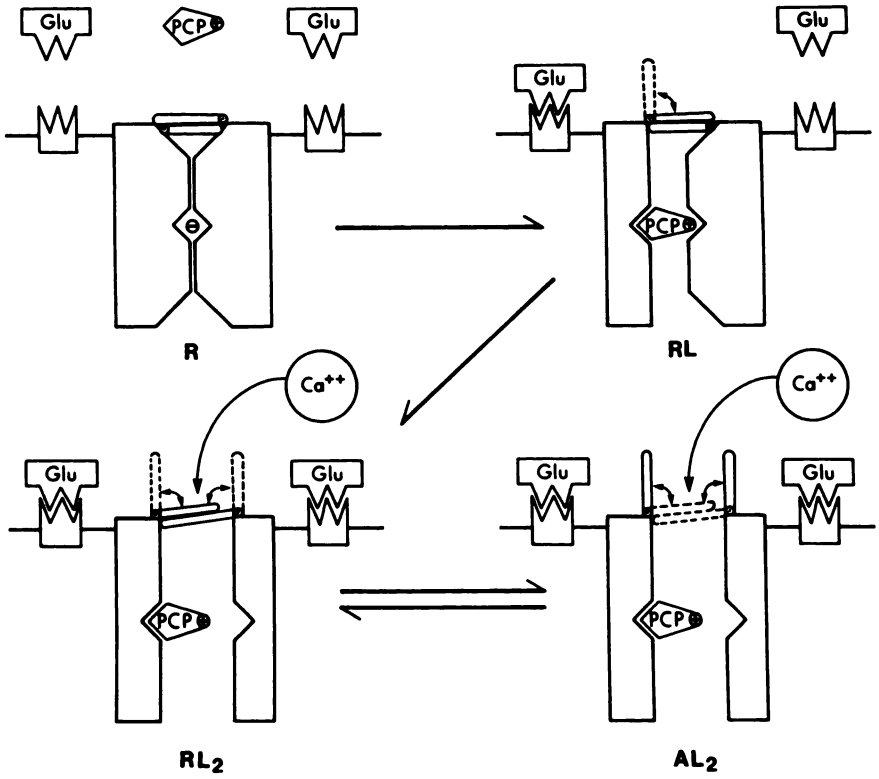

Fig. 4. Schematic model of NMDA receptor activation by L-glutamate. In the absence of L-glutamate (*GLU*), the NMDA channel remains in the closed conformation (*R*). This conformation does not permit ligand binding to the PCP receptor. Binding of a single molecule of L-glutamate induces a conformational change (*RL*) that does not allow complete channel opening but does allow binding of PCP receptor ligands following diffusion through a slow hydrophobic path. The slow path may represent diffusion through the closed channel gating mechanism or through hydrophobic regions of the single-agonist-associated conformation of the NMDA receptor complex. Binding of two molecules of L-glutamate induces a conformation (RL_2) that is in equilibrium with the open conformation (AL_2). The open conformation allows diffusion of PCP receptor ligands to their binding site via the open channel, leading to a fast association path. Ligand binding to the PCP receptor prevents the entry of ions such as Ca^{2+} through the open NMDA channel.

represents the activated (open) conformation of the complex. We further proposed that the resting conformation (*R*) does not permit [^{3}H]MK-801 association, whereas the agonist-associated resting conformations (*RL* and RL_2) permit slow association via a hydrophobic diffusion path that is independent of the open channel. The presence of distinct hydrophilic and hydrophobic association paths is consistent with reports that for other channels there are such independent diffusion paths for noncompetitive blockers whose pK_a values are near neutral pH (22, 23).

Our previous data were obtained at only extreme concentrations of L-glutamate and glycine. That work, therefore, did not examine the ability of the proposed model to account for the dose-response relationship between L-glutamate and NMDA receptor activation. In order to determine the ability of the proposed model to account for our present data, predicted relative values for steady state fast and slow binding were determined using the equations

$$[RL] = 2[R][L]/K_1 \quad (2)$$

$$[RL_2] = [RL][L]/2K_2 \quad (3)$$

$$[AL_2] = \Phi[RL_2] \quad (4)$$

in which K_1 and K_2 represent the affinities of L-glutamate binding to the *R* and *RL* conformations, respectively, and Φ represents the conformational equilibrium constant between closed (RL_2) and open conformations.

For initial simulations, values for total steady state binding and Φ were defined as the sum (87,160 cpm/mg of protein) and ratio (0.75), respectively, of slow and fast binding in the presence of 100 μM L-glutamate. In addition, the value for K_2 was assumed to equal that of K_1, corresponding to a situation in which there is no cooperativity of L-glutamate binding. For initial simulations, therefore, only a single free parameter (K_1) was manipulated to account for the variation in fast, slow, and total binding of [^{3}H]MK-801 at L-glutamate concentrations between 0.3 and 10 μM L-glutamate. Optimal correlations between predicted and observed values were obtained with an assumed affinity of 0.85 μM for L-glutamate binding, corresponding to an apparent EC_{50} of 1.7 μM for fast binding of [^{3}H]-MK-801. Under these conditions, the correlation coefficient (*r*) between predicted and observed values for total steady state binding was 0.99, indicating a highly significant correlation ($p < 0.001$). Significant correlations were observed as well between predicted and observed values for steady state binding that occurred with fast ($r = 0.95$) and slow ($r = 0.86$) kinetics (slow, $p < 0.05$; fast, $p < 0.005$).

Specific modifications were incorporated into the model in order to determine whether significant improvement could be obtained in the correlation between predicted and observed values. Receptor cooperativity was incorporated by assuming that the affinity of binding for the second molecule of L-glutamate (K_2) is significantly stronger than the affinity of binding for the first molecule (K_1). Desensitization was incorporated by assuming transitions from the *RL*, RL_2, and/or AL_2 states to corresponding desensitized states, which were postulated not to allow [^{3}H]MK-801 association. Subconductance states were incorporated by assuming the existence of activated conformations associated with fewer than 2 molecules of L-glutamate (24). None of these modifications improved the correlation between predicted and observed values.

Postulating the existence of a third binding site for L-glutamate led to correlation coefficients that were larger (slow, $r = 0.95$; fast, $r = 0.99$) but not significantly improved as compared with the proposed four-state model. The existence of a third binding site might correspond to the presence of a third subunit containing the agonist binding site within each functional receptor complex, as has been reported in the case of the

strychnine-sensitive glycine receptor (25). A similar nonsignificant increase in correlation coefficients (slow, $r = 0.94$; fast, $r = 0.99$) was obtained by postulating that the probability of receptor activation subsequent to agonist binding is greater in the presence of high as compared with low concentrations of L-glutamate (Fig. 3). Based upon the proposed model, in the presence of the three lowest concentrations of L-glutamate (0.3–1.0 μM), the apparent probability of receptor activation subsequent to agonist binding was 31 ± 6%, whereas, in the presence of the three highest concentrations (3–100 μM), the apparent probability was 56 ± 1%. The mechanism underlying such differential probability, however, remains to be determined.

The reaction scheme that accounts for the activation of NMDA receptors in the present study is similar to reaction schemes that account for activation of both nicotinic (26) and $GABA_A$ (27) receptors by their agonists (Fig. 4). This study, thus, provides further biochemical support for the hypothesis (28) that the NMDA receptor complex belongs to the Class 1 superfamily of ligand-gated channels.

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