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Structure-Activity Relationships for Amino Acid Transmitter Candidates Acting at *N*-Methyl-D-Aspartate and Quisqualate Receptors

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Dose-response curves for activation of excitatory amino acid receptors on mouse embryonic hippocampal neurons in culture were recorded for 15 excitatory amino acids, including the L-isomers of glutamate, aspartate, and a family of endogenous sulfur amino acids. In the presence of 3 μM glycine, with no extracellular Mg, micromolar concentrations of 11 of these amino acids produced selective activation of *N*-methyl-D-aspartate (NMDA) receptors. L-Glutamate was the most potent NMDA agonist (EC_{50} 2.3 μM) and quinolinic acid the least potent (EC_{50} 2.3 mM). Dose-response curves were well fit by the logistic equation, or by a model with 2 independent agonist binding sites. The mean limiting slope of log-log plots of NMDA receptor current versus agonist concentration (1.93) suggests that a 2-site model is appropriate. There was excellent correlation between agonist EC_{50}s determined in voltage clamp experiments and K_ds determined for NMDA receptor binding (Olverman et al., 1988). With no added glycine, and 1 mM extracellular Mg, responses to NMDA were completely blocked; responses to kainate and quisqualate were unchanged. Under these conditions, glutamate and the sulfur amino acids activated a rapidly desensitizing response, similar to that evoked by micromolar concentrations of quisqualate and AMPA, but mM concentrations of L-aspartate, homoquinolinic acid, and quinolinic acid failed to elicit a non-NMDA receptor-mediated response. Except for L-glutamate (EC_{50} 480 μM), the low potency of the sulfur amino acids prevented the study of complete dose-response curves for the rapidly desensitizing response at quisqualate receptors. Small-amplitude nondesensitizing quisqualate receptor responses were activated by much lower concentrations of all quisqualate receptor agonists. Full dose-response curves for the nondesensitizing response were obtained for 9 amino acids; L-glutamate was the most potent endogenous agonist (EC_{50} 19 μM). Domoate (EC_{50} 13 μM) and kainate (EC_{50} 143 μM) activated large-amplitude, nondesensitizing responses.

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In addition to L-glutamate and L-aspartate, the mammalian nervous system contains a number of other potent excitatory amino acids, including several sulfur amino acids (Do et al., 1986). In addition to their potential role as excitatory neurotransmitters, the agonist action of some of these compounds has been suggested to underlie various neurodegenerative diseases (see Choi, 1988, for a recent review); a rare, but well-established, example is the association of *S*-sulfo-L-cysteine with extensive brain damage in patients with cysteine oxidase deficiency (Olney et al., 1975).

The classification of excitatory amino acid receptors into subtypes selectively activated by *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate is now widely accepted (Watkins and Evans, 1981; Mayer and Westbrook, 1987), although the differentiation of kainate and quisqualate receptors is incomplete, and depends largely on differences in the distribution of high affinity kainate and quisqualate receptors in different brain regions (e.g., Monaghan et al., 1985). Although kainate and quisqualate produce physiological responses with different characteristics (Kiskin et al., 1986; Trussell et al., 1988; Mayer and Vyklicky, 1989), there is considerable evidence for interactions between kainate and quisqualate (Kiskin et al., 1986; O'Brien and Fischbach, 1986; Perouansky and Grantyn, 1989) that could occur if both ligands bind to a common receptor, but with differing efficacy as agonists, or with different susceptibility to desensitization. Responses to kainate are nondesensitizing and associated with low single-channel conductance (Ascher and Nowak, 1988; Cull-Candy and Usowicz, 1989), while quisqualate produces a rapidly desensitizing response, with higher single-channel conductance (Tang et al., 1989; Trussell and Fischbach, 1989). Because the agonists described in this paper produce rapidly desensitizing responses similar to those evoked by quisqualate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), a selective quisqualate receptor agonist, we will refer to these as quisqualate receptor responses (cf. Mayer and Vyklicky, 1989).

Although binding experiments have been used to measure the affinity of NMDA and quisqualate receptors for different amino acids, there have been no systematic attempts to study structure-activity relationships for activation of NMDA and quisqualate receptors using physiological techniques. This is important because binding experiments are usually performed at equilibrium, following long periods of incubation with ligands. In the case of nicotinic acetylcholine receptors, agonist affinities determined by this approach are very poorly correlated with agonist potency

determined in physiological experiments (e.g., Changeux et al., 1984). The most widely accepted explanation for this is that acetylcholine receptors in membrane preparations become desensitized during binding experiments, and that desensitized acetylcholine receptors have a much higher affinity for agonist than the resting state of the receptor at the intact neuromuscular junction, which binds agonist to produce a synaptic response. Differences between results from binding experiments and those obtained by physiological measurements of agonist potency could also arise from the experimental conditions used for each type of experiment. This is particularly true for the study of excitatory amino acids, because binding experiments are usually performed in solutions without divalent cations or sodium chloride, to prevent Na-, Ca-, and Cl-dependent uptake of amino acids into microsomes that form in the membrane preparations used for binding (e.g., Pin et al., 1984; Fagg and Lanthorn, 1985). From studies on several receptor species it is known that changes in the concentration of these ions can alter receptor affinity (e.g., Pert and Snyder, 1974; Birdsall et al., 1979). Also, binding studies do not discriminate among agonists, partial agonists, and antagonists. For these reasons, electrophysiological measurement of dose-response curves for activation of glutamate receptors by neurotransmitter candidates is of considerable interest.

Apart from the technical difficulties that arise during measurement of the dose-response curve for any agonist that binds to receptor-activated ion channels (these include the need to voltage-clamp large amplitude currents, as well as the problem of desensitization), the response of mammalian neurons to excitatory amino acids suffers from the additional problem that many amino acids can activate both NMDA and quisqualate receptors (e.g., Watkins and Evans, 1981; Mayer and Westbrook, 1984). Thus methods had to be developed that allowed study of the effects of mixed agonists on each receptor species in isolation. Although, in principle, selective NMDA and quisqualate antagonists could be used, several practical difficulties emerge, including the lack of complete selectivity of the available competitive antagonists for either NMDA or quisqualate receptors (e.g., Kleckner and Dingledine, 1989; Verdoorn et al., 1989), and the need for very high antagonist concentrations if, at the top of the agonist dose-response curve, displacement of antagonist is to be prevented.

In this paper we describe experiments on embryonic mouse hippocampal neurons in which the selective activation of NMDA and quisqualate receptors was studied without the use of antagonists. Dose-response curves were determined for a series of 15 excitatory amino acids, including all the known potential transmitter candidates, other endogenous amino acids of neuropathological importance, and some amino acid analogs of experimental interest.

Materials and Methods

Cell culture. Experiments were performed on primary dissociated cultures of mouse embryonic hippocampus. Nerve cell cultures were prepared by dissociating the hippocampi of 16–17 d old C57Bl/6 mouse embryos and plating the resulting cell suspension onto confluent hippocampal glial cell feeder layer cultures. The mothers were killed by cervical dislocation prior to removal of all the fetuses. The growth medium contained MEM, 5% horse serum, and a nutrient supplement containing transferrin, insulin, selenium, corticosterone, triiodothyronine, progesterone, and putrescine (Guthrie et al., 1987); no antibiotics were used. Complete details are given in Mayer et al. (1989).

Recording and perfusion techniques. Experiments were performed at room temperature (25–27°C) 7–14 d after cultures were plated. Voltage clamp was achieved using whole-cell patch clamp recording and an Axon instruments "Axoclamp 2" discontinuous amplifier set at a gain of 2–4 nA/mV, with a switching frequency of approximately 10 kHz. The series resistance was usually kept below 10 MΩ. The recording chamber was perfused at 0.5–1.0 ml/min with extracellular solution, and a flow pipe array similar to that described by Johnson and Ascher (1986) was used for faster perfusion around individual neurons as described below.

The fast perfusion system consisted of an array of 9 glass tubes (Yellen, 1982), each 400 μm in external diameter with wall thickness of approximately 30 μM; the array was mounted on a hydraulic manipulator driven by a stepping motor and positioned within 200 μM of neuronal cell bodies using a mechanical manipulator. A pump was used to drive solution through the tubes at approximately 150 μm/msec, and 3-way latching solenoid valves were used to direct the solution back to reservoirs or onto nerve cells. Cells were always bathed in a rapidly flowing stream of control solution, except during application of agonist. Rapid solution changes were achieved as follows: a barrel of the perfusion system containing control solution was positioned above the soma of a selected neuron, and the valve connected to this barrel opened to perfuse the area around the nerve cell; the stepper motor then moved the flow pipe such that an adjacent barrel containing an agonist was positioned above the neuron (traverse time 140 msec); on reaching this new position the valve controlling solution flow through the barrel previously centered above the soma was closed and, simultaneously, solution flow onto the neuron was started through the newly positioned barrel. In well-isolated neurons, with clearly defined dendritic trees, the solution exchange time constant was less than 10 msec, as measured using sodium ion concentration jumps in the presence of kainic acid (Vyklicky et al., 1990). The junction potential change measured with a cell-free patch electrode was faster, with a time constant less than 1 msec. The slower solution change recorded in whole cell experiments probably reflects perfusion of the much larger area of the dendritic tree of hippocampal neurons compared to faster exchange around the tiny opening of a patch pipette.

Experimental solutions. The extracellular solution contained (mM) 160 NaCl, 2.5 KCl, 2 or 0.2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 0.01 mg/ml phenol red; pH was adjusted to 7.3 with NaOH. Tetrodotoxin (400 nM) was added to block action potentials and synaptic activity, and bicuculline methochloride (5 μM) added to block spontaneous inhibitory postsynaptic potentials. Mg was omitted from solutions containing agonist during NMDA receptor experiments to avoid ion channel block. Glycine, excitatory amino acids, and the glycine antagonist 7-chlorokynurenic acid were added to the extracellular solution as required. Pipettes for whole-cell recording contained (mM) 125 $CsMeSO_3$, 15 CsCl, 10 HEPES, 5 CsBAPTA, 0.5 $CaCl_2$, 3 $MgCl_2$, and 2 MgATP; pH was adjusted to 7.2 with CsOH and osmolarity to 305 mosM with sucrose if necessary. Salts, biochemicals, excitatory amino acids and antagonists were purchased from Aldrich, Diagnostic Chemicals, Molecular Probes, Sigma, and Tocris Neuramin.

Results are presented as mean ± SD.

Results

Selective activation of NMDA and quisqualate receptors by mixed agonists

In our initial experiments we attempted to determine conditions that would allow selective activation of either NMDA or quisqualate receptors by agonists with activity at both subtypes of glutamate receptor. This was necessary because in our experiments neurons always responded to both NMDA and quisqualate. To allow selective activation of quisqualate receptors we used solutions containing 1 mM Mg, 300 nM of the glycine antagonist 7-chlorokynurenic acid, with no added glycine (background contamination expected to be 20–50 nM glycine), and held the membrane potential at −60 mV. This effectively eliminated the response to NMDA but did not alter responses to quisqualate or kainate (Fig. 1). All subsequent experiments, on both NMDA and quisqualate receptors, were performed at −60 mV.

With no added Mg, 3 μM glycine, and 0.2 mM Ca, we recorded large NMDA receptor responses that were weakly or nondesen-

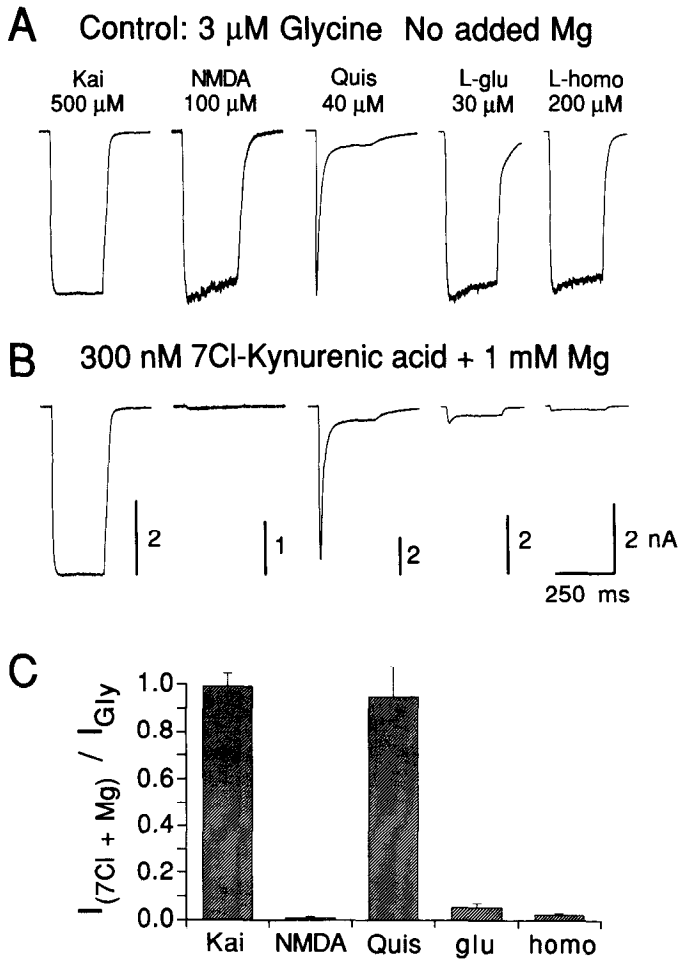

Figure 1. Selective activation and block of NMDA receptors. *A,* Responses to fast application of kainate (*Kai*), *NMDA,* quisqualate (*Quis*), L-glutamate (*L-glu*), and L-homocysteate (*L-homo*), under conditions permissive for NMDA receptor activation (3 μM glycine, no added Mg). At the concentrations used, NMDA receptor activation by L-glutamate and L-homocysteate is close to maximal. *B,* Responses of the same cells after block of NMDA receptor-mediated responses by 1 mM Mg and 300 nM 7-chlorokynurenic acid. Residual responses to L-glutamate and L-homocysteate are due to activation of quisqualate receptors. *C* summarizes the effect of NMDA receptor block on the peak amplitude of responses to the above agonists, recorded from a minimum of 6 cells (24 observations); error bars indicate 1 SD.

sitizing, and thus relatively simple to study. In contrast, quisqualate receptor responses were strongly desensitizing under all experimental conditions. This was a disadvantage for the study of concentration-response relationships, but unfortunately there is no method we know of that completely blocks desensitization of responses at quisqualate receptors on mammalian CNS neurons. Treatment with concanavalin-A reduces but never eliminates desensitization (Mayer and Vyklicky, 1989) and has the disadvantage that concanavalin-A-treated neurons become fragile and difficult to study with patch electrodes. In addition, it is not known how concanavalin-A acts, or whether it modifies the kinetics of activation of the quisqualate receptor.

Selective activation of NMDA receptors by mixed agonists was possible because activation of quisqualate receptors was negligible at doses of amino acid that produced full activation of NMDA receptors. Evidence for this was obtained by examining the response of individual neurons to the highest concentrations of L-glutamate and L-homocysteate used for construction of NMDA receptor dose-response curves (see below), under conditions that were either permissive for, or that blocked, activation of NMDA receptors (Fig. 1). The difference in rise time of NMDA- and quisqualate-receptor-mediated currents, and the rapid onset of desensitization of responses at quisqualate receptors, limited the contribution of quisqualate receptors to the peak response under conditions permissive for NMDA receptor activation to only 5.4 ± 1.9% of the total response to 30 μM L-glutamate, and to 2.4 ± 0.6% of the total response to 200 μM L-homocysteate (24 observations on 7 cells per agonist). The selective activation of NMDA versus quisqualate receptors obtained with low doses of L-glutamate and L-homocysteate (Fig. 1) was a general feature of the action of the mixed agonists studied in the experiments described here because all agonists had substantially lower affinity for activation of quisqualate versus NMDA receptors. The breakthrough of quisqualate receptor current will be even smaller at concentrations lower than those required for saturation of NMDA receptor activation; therefore, no correction for quisqualate receptor current was made during analysis of NMDA receptor dose-response curves.

Structure-activity relationships for NMDA receptors

Ligand binding experiments with the NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (^{3}H-D-AP5) have revealed over 35 compounds with affinity constants for NMDA receptors of less than 100 μM (Olverman et al., 1988). We chose to examine L-glutamate and L-aspartate; their sulfonic and sulfinic analogs L-homocysteate, L-homocysteinesulfinate, L-cysteate, and L-cysteinesulfinate; the tryptophan metabolite quinolinic acid and its longer-chain analog homoquinolinic acid, *S*-sulfo-L-cysteine, L-serine-*O*-sulfate; and NMDA itself. All were good agonists and evoked large NMDA receptor currents, though as would be expected from the results of binding experiments with ^{3}H-D-AP5, differences in their potency covered a 1000-fold range of equieffective concentrations. Structures of these compounds, grouped into aspartate- and glutamate-like analogs (see below), are illustrated in Figure 2.

Concentration-response curves for individual neurons were constructed using a series of 6 sequential doses of amino acid, spanning a 2.5 log unit concentration range, each dose repeated in duplicate, and usually 2 runs through the concentration range completed per neuron. Each run was analyzed individually. Preliminary experiments with L-glutamate, L-aspartate, L-homocysteate, and L-homocysteinesulfinate, to determine the appropriate concentration range for activation of NMDA receptors in physiological experiments, revealed that concentrations could be chosen by assuming that the K_ds determined by displacement of ^{3}H-D-AP5 binding (Olverman et al., 1988) were approximately 3 times lower than the concentrations required for 50% activation of the NMDA receptor current in voltage-clamp experiments (L. Vyklicky and M. L. Mayer, unpublished observations). This empirical relationship was used to establish appropriate concentration ranges for the other agonists examined in this study. Examples of NMDA receptor responses to L-glutamate, L-aspartate, and quinolinate are shown in Figure 3.

To allow comparison of results from our experiments with other studies on the concentration-response relationship for activation of NMDA receptors (Kushner et al., 1988; Verdoorn and Dingledine, 1988; Priestley et al., 1989), dose-response curves were fitted to the logistic equation

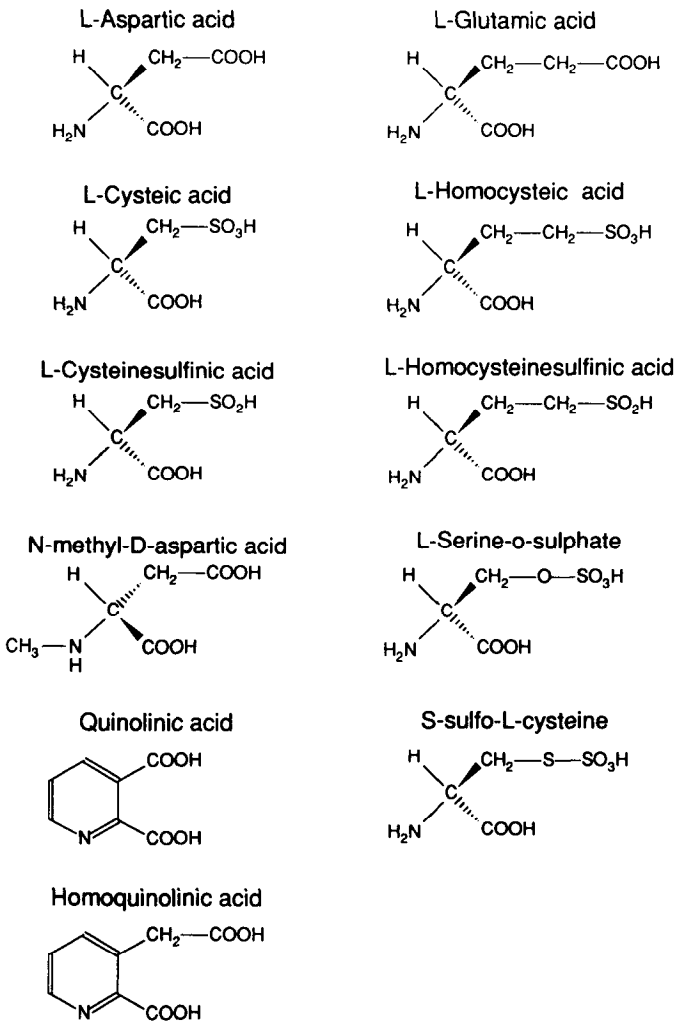

Figure 2. Agonists used to study NMDA and quisqualate receptor structure-activity relationships. The 2 columns are arranged into short-chain (aspartate-like) and long-chain (glutamate-like) compounds. Abbreviations for these 11 compounds used in subsequent figures are: L-glutamate, *L-glu*; L-aspartate, *L-asp*; L-homocysteic acid, *L-homo*; L-homocysteinesulfinic acid, *L-HCSA*; L-cysteic acid, *L-cys*; L-cysteinesulfinic acid, *L-CSA*; L-serine-*O*-sulfate, *L-SOS*; *N*-methyl-D-aspartic acid, *NMDA*; *S*-sulfo-L-cysteine, *S-cys*; quinolinic acid, *Quin*; and homoquinolinic acid, *Homoquin*.

$$I = I_{max} \times \frac{1}{1 + \left\{\frac{EC_{50}}{[dose]}\right\}^{n}}$$

in which I is the amplitude of the current response at a given dose of agonist, I_{max} is the response to a saturating dose of agonist, EC_{50} is the concentration of agonist that produces 50% of the maximum response, and n is a factor that describes the steepness of the dose-response curve and is usually termed the Hill coefficient. Good fits were obtained with the logistic equation for all the agonists examined in our experiments (Fig. 3). EC_{50}s and the Hill coefficient obtained from the logistic equation for the 11 amino acids examined in our experiments on NMDA receptors are listed in Table 1. The NMDA receptor dose-response curve was steeper than predicted by a single binding site absorption isotherm for all agonists. The mean value for n was 1.4 ± 0.16.

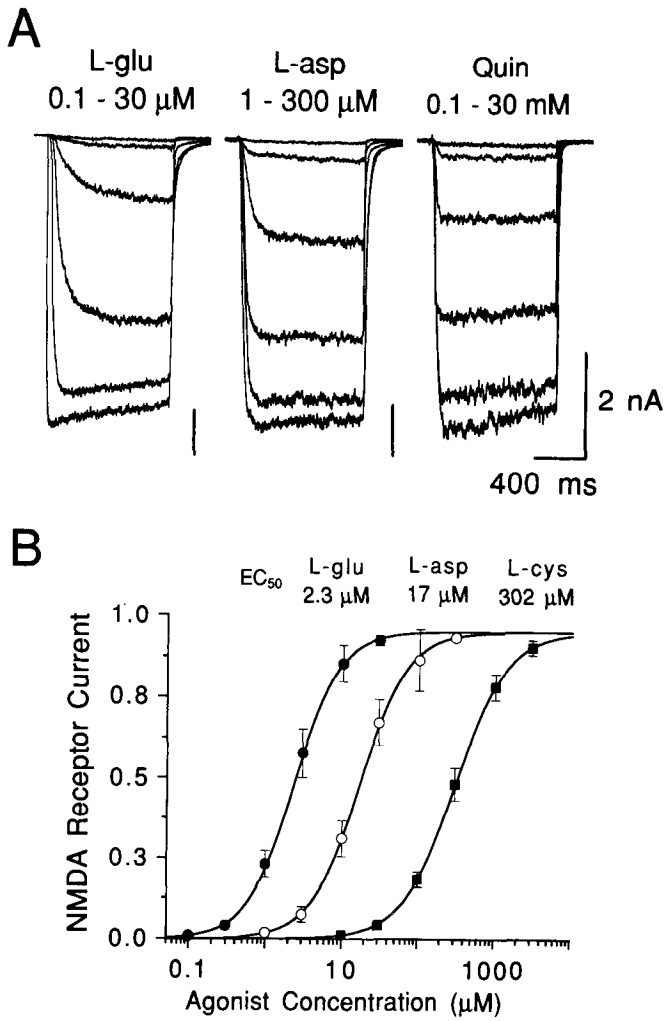

Figure 3. Dose-response curves for NMDA receptor activation. *A*, Examples of responses to L-glutamate, L-aspartate, and quinolinate at the concentrations indicated above each set of traces. Responses were recorded from 3 neurons and for display purposes are scaled to have similar maximum amplitude. *B*, Analysis of similar responses for L-glutamate, L-aspartate, and L-cysteate. Dose-response curves recorded in individual neurons were normalized with respect to the maximum current (I_{max}) derived from analysis using the logistic equation. The results of this analysis were pooled for illustrative purposes; each data point is the mean ± SD of 10–19 observations from 5–11 cells. The concentration calculated for 50% of maximal activation (EC_{50}) and indicated above each dose-response curve was obtained by analysis of the pooled data.

Consistent with results from ligand binding experiments, L-glutamate was the most potent, and quinolinic acid the least potent, NMDA receptor agonist of the 11 compounds tested. The potency sequence was exactly that predicted by binding experiments, with the exception that L-aspartate (nearly twice as potent as predicted) changed rank with L-homocysteinesulfinate. The potency sequence at NMDA receptors was: L-glutamate > *S*-sulfo-L-cysteine > L-homocysteate > L-aspartate > homoquinolinate ≥ L-homocysteinesulfinate ≥ NMDA > L-cysteinesulfinate ≥ L-serine-*O*-sulfate > L-cysteate > quinolinate (≥ indicates K_ds not significantly different, *t*-test, $p > 0.10$). In the only other study of NMDA receptor agonist potency, in which EC_{50}s were determined for L-glutamate, L-aspartate, and NMDA, the values obtained were similar to those

Table 1. NMDA receptor structure-activity relationships

Agonist	Logistic equation[a]		2-site model[a]	Binding[b]
	EC_{50} (μM)	n	K_d (μM)	K_d (μM)
L-Glutamate	2.3 ± 0.6	1.5 ± 0.14	1.1 ± 0.2	0.9
S-sulfo-L-cysteine	8.2 ± 1.2	1.4 ± 0.13	3.7 ± 0.5	2.1
L-Homocysteate	12.9 ± 4.7	1.3 ± 0.14	5.3 ± 1.5	3.9
L-Aspartate	16.9 ± 3.7	1.5 ± 0.16	7.5 ± 1.7	11
Homoquinolinate	26.2 ± 3.9	1.5 ± 0.07	12.4 ± 1.8	7.1
L-HCSA	29.9 ± 7.8	1.4 ± 0.13	13.3 ± 3.1	10
NMDA	34.9 ± 9.2	1.4 ± 0.24	16.4 ± 3.5	11
L-CSA	43.0 ± 8.0	1.5 ± 0.09	19.7 ± 4.0	13
L-Serine-*O*-sulfate	44.7 ± 11.4	1.4 ± 0.15	18.8 ± 5.1	17
L-Cysteate	302 ± 47.0	1.3 ± 0.10	138 ± 27	120
Quinolinate	2311 ± 825	1.3 ± 0.12	919 ± 282	230

NMDA receptor EC_{50}s and Hill coefficients (n), obtained using the logistic equation; microscopic K_ds, obtained using a 2-site model, with independent binding (equation 1); binding K_ds estimated using displacement of ^{3}H-D-AP5, for a series of 11 NMDA receptor agonists. Values are the means from analysis of dose-response curves recorded in individual neurons.

[a] Values are mean ± SD of 10–19 observations from 5 to 11 cells.

[b] Binding data from Olverman et al., 1988.

reported here, with L-aspartate being more potent than NMDA (Verdoorn and Dingledine, 1988).

Because the potency of the above NMDA receptor agonists spans a 1000-fold range of EC_{50}s, we considered the possibility that low affinity agonists might be partial agonists. In experiments in which saturating doses of L-glutamate, L-aspartate, L-homocysteate, L-cysteate, and quinolinate were applied to the same cell, all agonists evoked similar amplitude responses. Thus, despite their low potency, cysteic and quinolinic acids are full agonists at NMDA receptors. The low potency of quinolinate is consistent with previous work (Peters and Choi, 1987).

The low potency of some agonists, especially L-cysteic acid and quinolinic acid, raises concerns that the action of these ligands could be due to contamination with a more potent agonist. If these compounds were inactive, and their response at NMDA receptors was due entirely to contamination with glutamate, we calculate, based on EC_{50} ratios, that our samples of cysteic and quinolinic acid would need to contain 0.76% and 0.1% glutamate, respectively, to produce the required response at NMDA receptors. But independent of tests for purity, we have evidence that contamination with glutamate *does not* underlie the agonist action of cysteic acid and quinolinic acid in our experiments, based on the different kinetics of responses to low doses of amino acid. As illustrated in Figure 3, responses to low doses of L-glutamate show slow activation kinetics, consistent with the high potency of this ligand. The fast decay of the responses illustrated in Figure 3 is due to rapid perfusion of agonist-free control solution containing 1 mM Mg. In preliminary experiments with Mg-free control solutions we have observed that the rate of decay of NMDA receptor current following fast termination of agonist application is inversely correlated with agonist potency, as would be expected if changes in agonist dissociation rate constant made significant contributions to determining potency. This effect is likely to underlie the slow kinetics of NMDA receptor activation recorded with low concentrations of potent agonists such as L-glutamate and *S*-sulfo-L-cysteine; in contrast, fast dissociation of agonists with low potency would contribute to fast activation kinetics. Responses to low doses of quinolinate and L-cysteate showed fast activation kinetics. If the response to these agonists was due to contamination with L-glutamate, then, at the foot of the dose-response curve, activation of NMDA receptors should also have occurred with slow kinetics, similar to those seen with L-glutamate.

A two-site independent subunit model for activation of NMDA receptors

As would be expected from the above potency rank order, plots of EC_{50}s determined in our voltage clamp experiments versus K_ds for displacement of the NMDA receptor antagonist AP5 determined in binding experiments by Olverman et al. (1988) showed high correlation ($r = 0.97$), but with a systematic 3-fold lower affinity for voltage clamp EC_{50}s than K_ds from binding experiments (Table 1). On average, excluding values for aspartate and quinolinate which lie off the correlation plot, voltage clamp EC_{50}s were 3.13 ± 0.49 times lower affinity than K_ds measured by displacement of AP5.

Because the results from binding and voltage clamp experiments showed such excellent correlation, we considered possible reasons for this systematic 3-fold difference in agonist potency. In addition to unknown effects of the ionic composition of the extracellular solution on ligand affinity (as discussed in the introductory remarks), it is possible that the logistic equation used to fit the NMDA receptor concentration-response relationship is not an accurate model for NMDA receptor activation. The logistic equation is a "high facilitation" model for agonist action and implies interactions between receptor subunits when the receptor channel complex has multiple agonist binding sites. Noninteger values >1 for n, as obtained from analysis of responses to NMDA receptor agonists, imply multiple binding sites but do not accurately determine the number of agonist binding sites or their strength of interaction. To confirm that activation of NMDA receptors requires the binding of more than 1 molecule of agonist, and to determine the stoichiometry of the reaction, we examined the limiting slope for the concentration-response relationship for low doses of NMDA plotted on a log-log scale, with agonist doses increasing by a factor of 2 (e.g., Bean, 1990). The result of this experiment, illustrated in Figure 4, showed clearly that the activation of NMDA re-

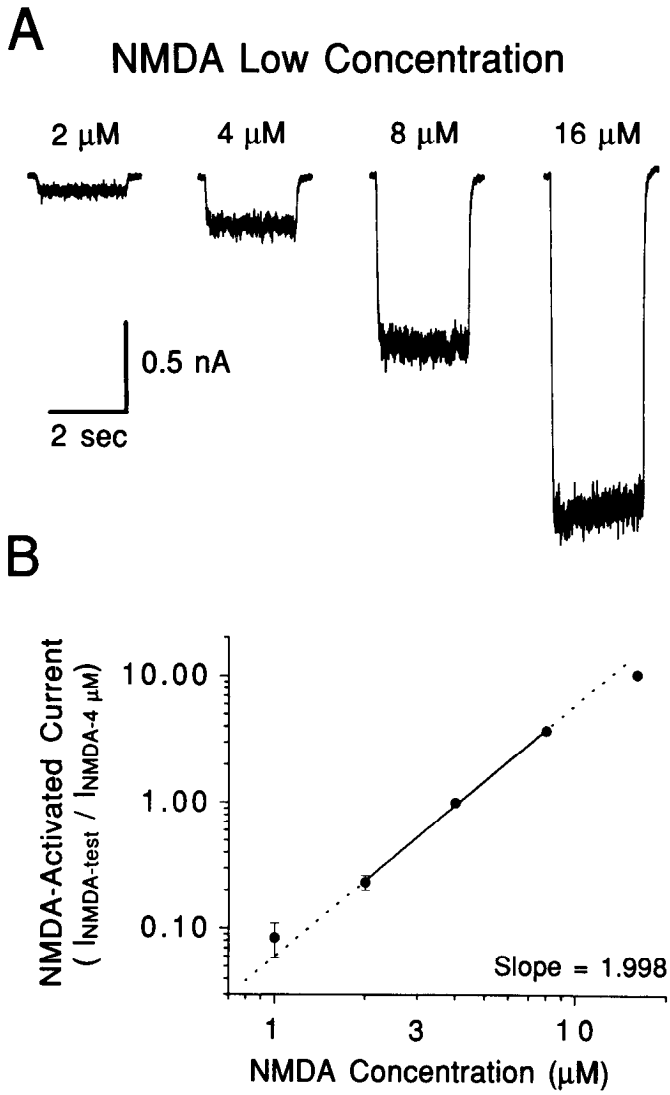

Figure 4. NMDA receptor activation at low concentrations of agonist. *A*, Responses to 4 concentrations of NMDA (increasing by a factor of 2) over the range 2–16 μM. *B*, Analysis of similar experiments performed on 6 neurons. Responses were normalized with respect to the current evoked by 4 μM NMDA; error bars indicate ± 1 SD. The *solid line* is a least-squares fit to data points over the range 2–8 μM and has a slope of 1.998; the *dotted line* is an extrapolation of the fitted curve over the range 1–16 μM. The slope of 1.998 shown here is that of the mean response of 6 cells, whereas the value given in the text is the mean of the slopes individually derived for each cell. Deviation of the data point at 1 μM was most likely due to inaccuracy in measuring the small amplitude of the response to NMDA; deviation from the fitted line is expected at concentrations of 16 μM and higher because the curve tends to zero slope at high concentrations of agonist.

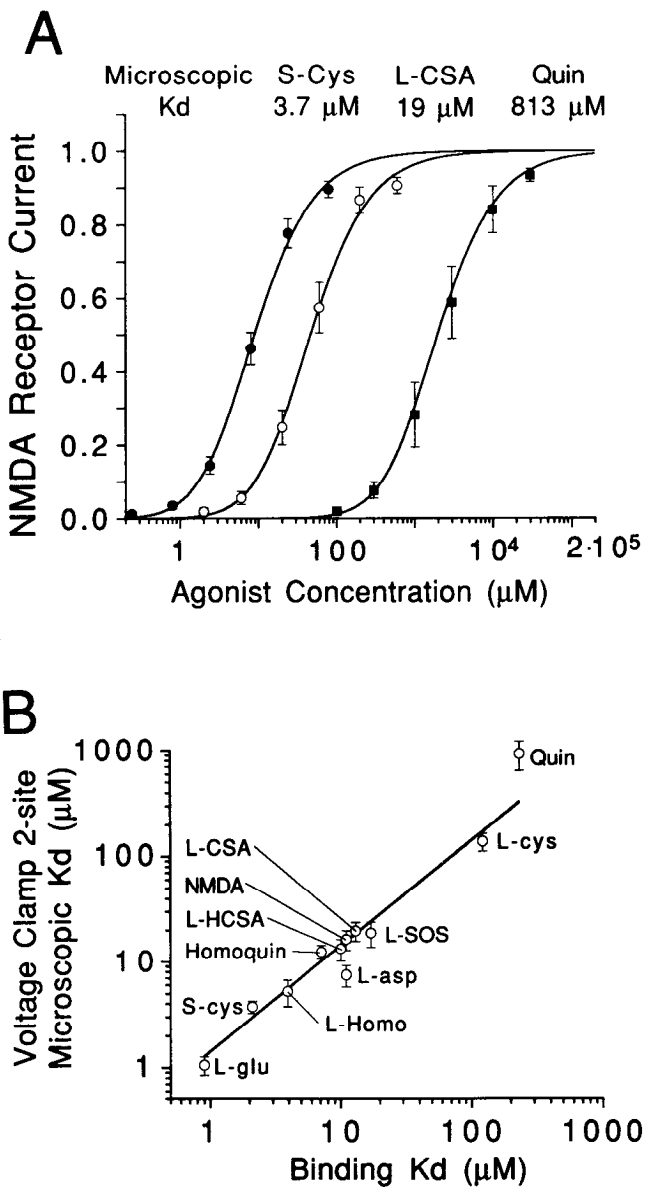

Figure 5. Analysis of NMDA receptor dose-response curves using a 2-site model, with independent binding of agonist. *A*, Mean dose-response curves for *S*-sulfo-L-cysteine, L-cysteinesulfinic acid, and quinolinic acid were derived from analysis of responses recorded in individual neurons using equation 1 and normalized with respect to the maximum current (I_{max}) estimated from the curve-fit analysis. Each data point is the mean ± SD of 11–15 observations from 6–8 cells. Microscopic K_ds indicated above each trace were obtained from curve fits to pooled data. *B*, The microscopic K_ds for 11 amino acids are plotted versus the K_ds estimated by Olverman et al. (1988) using displacement of ^{3}H-D-AP5. The fit is by linear regression, weighting data points inversely with their standard deviation.

ceptors has a steeper concentration-response relationship at low doses of agonist than would be predicted if only a single molecule of NMDA was required to activate the receptor channel complex. The limiting slope of the log-log plot over the NMDA concentration range 2–8 μM had a mean value of 1.93 ± 0.27 (n = 6), suggesting a stoichiometry of 2.

Our data for NMDA receptor agonists were very well fit by a model for a receptor with 2 independent agonist binding sites in which A_2R^* is the activated state of the receptor (e.g., Colquhoun and Ogden, 1988; Bean, 1990):

$$A + R \underset{k_{off}}{\overset{2k_{on}}{\rightleftharpoons}} AR + A \underset{2k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A_2R^*$$

$$\text{in which } I = I_{max} \times \frac{C^2}{1 + 2C + C^2} \quad (1)$$

where C is the agonist concentration divided by the microscopic equilibrium affinity constant for an individual agonist binding site ($K_d = k_{off}/k_{on}$, where k_{off} and k_{on} are the dissociation and association rate constants for agonist binding). Fits obtained

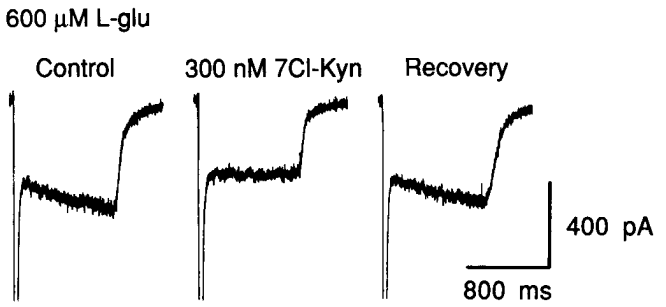

Figure 6. NMDA receptor activation at high concentrations of agonist interferes with the measurement of sustained responses at quisqualate receptors. The control and recovery traces show responses to 600 μM L-glutamate, almost 300× the NMDA receptor EC_{50}, but with 1 mM Mg and no added glycine. The response consists of a rapidly decaying peak, followed by a slow increase in current. The peak of the rapidly desensitizing response evoked at quisqualate receptors is truncated at the gain used for display. The slow inward-current relaxation was blocked by low concentrations of the glycine antagonist 7-chlorokynurenic acid (*7Cl-Kyn, middle trace*), leaving a sustained response similar to that activated by quisqualate and AMPA. Similar slow responses were evoked by high concentrations of aspartic acid (not shown). The holding potential was −60 mV.

using this model and those obtained using the logistic equation were virtually indistinguishable (cf. Figs. 3 and 5).

For the 11 NMDA receptor agonists examined in the present study, a plot of the K_d values determined by this 2-site model versus K_ds determined in binding experiments (Fig. 5*B*) showed strong correlation similar to that obtained by analysis using the logistic equation, but K_ds determined with a 2-site model were on average only 1.4 ± 0.25 times lower affinity than those measured in binding experiments (Table 1). A model with 3 independent binding sites also gave good fits to dose-response curves for NMDA receptor agonists, but yielded microscopic K_ds of higher affinity than obtained in binding experiments and was not supported by data at low concentrations of agonist (Fig. 4).

Structure-activity relationships for rapidly desensitizing responses at quisqualate receptors

Experiments on quisqualate receptors were performed in solutions containing no added glycine and 1 mM Mg, to block activation of NMDA receptors. We also added a 300 nM concentration of the glycine antagonist 7-chlorokynurenic acid because our experimental solutions were expected to contain a background contamination of around 20 nM glycine, which is sufficient to allow partial activation of a slowly desensitizing response to NMDA. The use of 7-chlorokynurenic acid was particularly important in measuring the equilibrium response at quisqualate receptors to high concentrations of agonists with NMDA receptor activity, because, for reasons not explained by recent theories describing the action of glycine on NMDA receptors, we have found that NMDA receptor agonists appear to produce activation of NMDA receptors in the absence of experimentally added glycine, but with very slow activation kinetics. Because the maximum amplitude of equilibrium responses to quisqualate receptor agonists is usually less than 10% of the maximal response that can be evoked by saturating concentrations of NMDA, breakthrough of NMDA receptor current was a potentially serious complication of analyzing steady-state quisqualate receptor responses evoked by mixed agonists. This

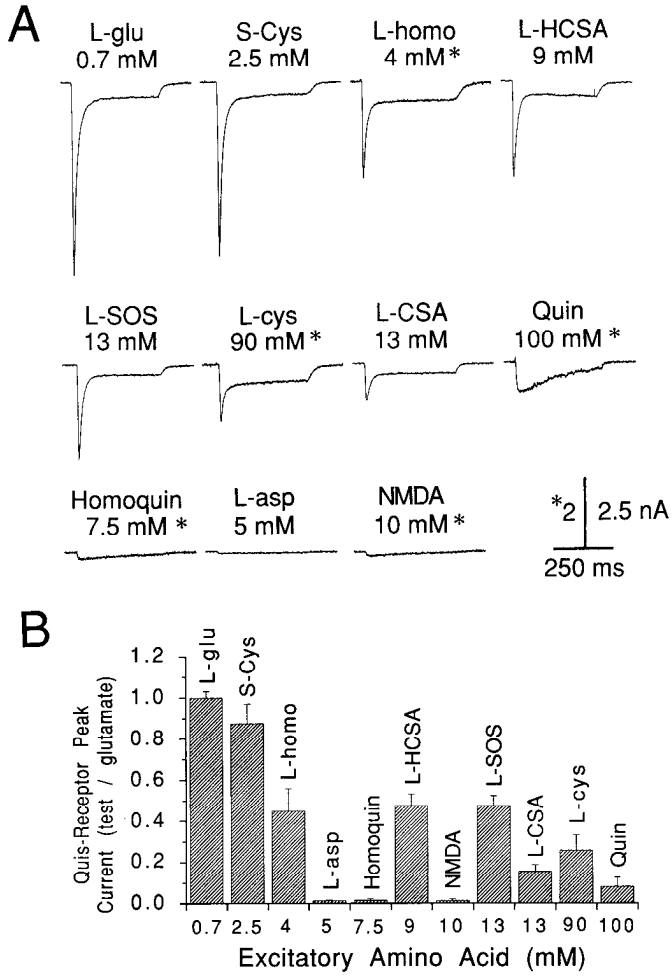

Figure 7. Activation of rapidly desensitizing quisqualate receptor responses by high concentrations of mixed agonists. *A*, Responses to 11 amino acids recorded with 1 mM Mg, no added glycine, and 300 nM 7-chlorokynurenic acid to block NMDA receptor activation. Amino acid concentrations are 300 times those required for 50% activation of NMDA receptors. Traces are from 2 neurons, as indicated by the current calibration, and are plotted so that the peak amplitude response of each neuron to L-glutamate is of equal size. *B* illustrates the mean result of similar experiments on 4 or 5 neurons (16–20 observations) for each group of agonists (responses to L-glutamate plus 5 other agonists were recorded in each cell). Responses are normalized with respect to those evoked by L-glutamate.

breakthrough of NMDA receptor current was blocked by low doses of 7-chlorokynurenic acid (Fig. 6), or by high concentrations of CPP, a selective, competitive NMDA receptor antagonist. The 300 nM concentration of 7-chlorokynurenic acid used in our experiments is too low to produce detectable antagonism of responses to kainate in *Xenopus* oocytes injected with brain messenger RNA, where the K_d for binding of 7-chlorokynurenic acid to kainate receptors has been estimated at 14 μM (Kleckner and Dingledine, 1989).

Responses to quisqualate and AMPA, the latter now widely considered the prototypic selective agonist for the quisqualate receptor coupled to ion channels, consist of an initial rapidly desensitizing current, followed by a sustained current. The ratio of initial/sustained current increases markedly with doses of quisqualate and AMPA (Mayer and Vyklicky, 1989), and the sustained current is selectively activated at low doses of agonist.

The most characteristic feature of responses to higher doses of quisqualate and AMPA is the speed of decay of the initial response, which in whole cell records can reach a time constant of <10 msec. Under conditions that block activation of NMDA receptors, NMDA, L-aspartate, homoquinolinic acid, and quinolinic acid failed to elicit a non-NMDA receptor-mediated response. L-Glutamate and the sulfur amino acids produced strong activation of a rapidly desensitizing response (Fig. 7*A*) similar to that evoked by micromolar concentrations of quisqualate and AMPA, but only when applied at mM concentrations, except for L-glutamate, which produced large quisqualate-like responses at concentrations of several hundred μM.

A rank order of potency for activation of quisqualate receptors was obtained by recording the amplitude of rapidly desensitizing quisqualate receptor responses, evoked by the series of 11 amino acids, when applied at a concentration 300 times that required for 50% activation of NMDA receptors (Fig. 7*B*). With these concentrations, activation of quisqualate receptors was submaximal for all agonists and considerably less than 50% of maximum for the weaker agonists. The potency sequence for the peak current response at quisqualate receptors was similar to that for activation of NMDA receptors and decreased in the order L-glutamate > *S*-sulfo-L-cysteine > L-homocysteate > L-homocysteinesulfinate > L-serine-*O*-sulfate > L-cysteinesulfinate > L-cysteate.

Responses to quinolinic acid (100 mM) were slower in time course than those evoked by the other excitatory amino acids and decayed to a sustained current with a time constant of 197 ± 45 msec (16 observations on 4 cells). In contrast, responses in the same cells to another low potency agonist, cysteic acid (90 mM), desensitized rapidly (time constant 14.1 ± 2.4 msec), as did responses to lower doses of the more potent quisqualate receptor agonists, which produced small responses of comparable amplitude to those activated by 100 mM quinolinic acid. Because the high concentrations of cysteic and quinolinic acids needed for activation of quisqualate receptors required the preparation of extracellular solutions in which sodium salts of these amino acids were isosmotically substituted for NaCl, we were concerned that the response to quinolinic acid, because of its different time course, might be artifactual and perhaps related to chloride concentration jumps (from 169 to 61 mM) during the application of this agonist. To control for this, we made fast applications of disodium succinate, a dicarboxylic acid structurally related to aspartic acid, but lacking an α-amine group. Application of 80 mM succinate to 3 cells (which produced a chloride concentration jump from 169 to 8.5 mM) did not produce any detectable current, although in the same neurons 600 μM L-glutamate evoked peak and sustained quisqualate receptor currents of 4.55 ± 0.97 and 0.18 ± 0.07 nA, respectively. In view of the extremely low potency of quinolinic acid, we did not further investigate the nature of the response to this agonist.

Attempts to measure the peak current response at quisqualate receptors were complicated by 2 factors: the extremely rapid onset of desensitization, and the low potency of many agonists, as judged by the experiment described above in which amino acids applied at concentrations 300 times their NMDA receptor EC_{50} (e.g., 90 mM for L-cysteic acid) failed to produce full activation of the rapidly desensitizing response at quisqualate receptors. Because of this we measured only the concentration-response curve for L-glutamate (Fig. 8). In 5 neurons the peak response to L-glutamate was well fit by the logistic equation, with an EC_{50} of 482 ± 98 μM, n = 1.1 (mean ± SEM). Results

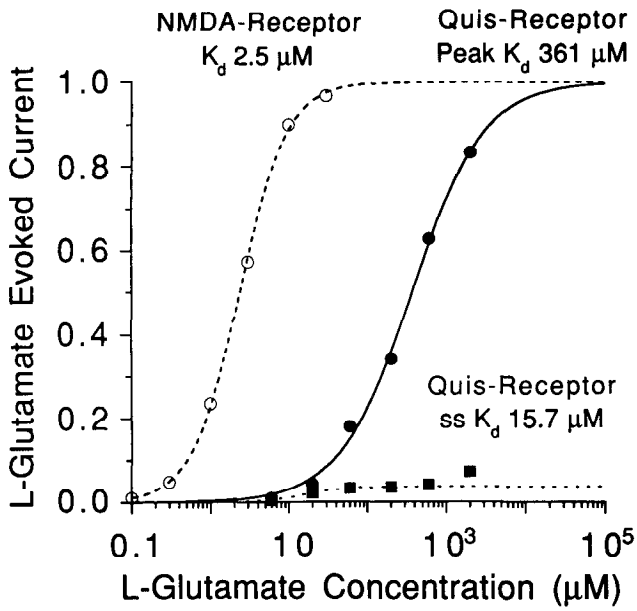

Figure 8. Dose-response curves for glutamate activation of quisqualate and NMDA receptors. The *solid line* is a fit of the logistic equation to the rapidly desensitizing response at quisqualate receptors (●) and has an EC_{50} of 361 μM. The data points are normalized with respect to the maximum current estimated from the logistic equation. The equilibrium response at quisqualate receptors (■) is plotted on the same scale and illustrates the higher affinity (EC_{50} = 15.7 μM) and lower amplitude of the sustained response. The fit of the equilibrium response dose-response curve is good over the range 2–600 μM (*dashed curve*), but cannot be seen at this scale (cf. Fig. 9); the point at 2 mM L-glutamate lies off the fitted curve due to activation of NMDA receptors (see Fig. 6). The *dashed curve* to the left is drawn using values obtained for activation of NMDA receptors on another cell by L-glutamate and illustrates the much higher affinity of NMDA receptors for L-glutamate.

shown in Figure 7 suggest that quisqualate receptor EC_{50}s for the other agonists tested in our experiments would be considerably lower affinity, although we cannot be certain that saturating concentrations of amino acids with very low affinity (e.g., L-cysteic acid) can evoke full activation of quisqualate receptors.

Equilibrium responses at quisqualate receptors

The nature of the sustained response to excitatory amino acids recorded after block of NMDA receptors is uncertain and could represent activation of one or several glutamate receptor subtypes. However, because, in all previous work performed to examine agonist structure-activity relationships at non-NMDA receptors, the speed of agonist application was not sufficiently fast to allow the study of the rapidly desensitizing response at quisqualate receptors, quantitative studies of the sustained response are of interest for comparison with previous work. In addition, if the sustained response does in fact represent activation of a receptor species distinct from that underlying the rapidly desensitizing response to quisqualate and L-glutamate, then measurements of agonist concentration-response curves are of considerable importance.

In our experiments, sustained, small-amplitude, non-NMDA receptor responses, similar to those evoked by 0.1–0.3 μM quisqualate and 1–3 μM AMPA, were also evoked by 7 of the mixed agonists tested in this series of experiments. The amino acid concentrations required for activation of the sustained quisqualate receptor response were lower, by at least a factor of 10,

than those required for activation of the rapidly desensitizing response, and complete concentration-response curves were obtained for all 7 mixed agonists, plus quisqualate and AMPA (Fig. 9). Analysis of these experiments was complicated by 2 opposing mechanisms activated by amino acid concentrations near the top of the dose-response curve for sustained quisqualate receptor currents. The first was slow activation of a second conductance mechanism, most likely the NMDA receptor, which generated additional current beyond that expected from activation of quisqualate receptors. This was particularly true for L-serine-*O*-sulfate and probably reflects weak agonist action of this compound at the glycine binding site on NMDA receptors. This would be consistent with the structure-activity relationships for glycine receptor activity described by McBain et al. (1989). The second mechanism, a reduction in agonist-evoked current with an increase in agonist concentration, was most apparent with quisqualate and AMPA, as has been noted previously (Ishida and Neyton, 1985; Perouansky and Grantyn, 1989). With the mixed agonists described here, this effect was quite small, and variable from cell to cell, perhaps because it was masked by activation of a second conductance mechanism as described above. To avoid the above issues, when the dose-response curve for sustained responses at quisqualate receptors showed either upward or downward deflections at near-saturating doses of agonist, these points were excluded from the curve fit. As previously described for NMDA receptors, sustained responses at quisqualate receptors for all 9 agonists were well fit both by the logistic equation (Hill coefficient mean = 1.34 ± 0.20) and by a model with 2 independent agonist binding sites, the latter giving microscopic K_d estimates of higher affinity than the EC_{50} values obtained by analysis with the logistic equation (Table 2).

In comparison to NMDA receptors, the structural features that determine ligand affinity at quisqualate receptors have yet to be defined at the same level of detail. Quisqualate receptor K_ds have been estimated from displacement of ^{3}H-AMPA binding (Murphy and Williams, 1987) for the 9 amino acids for which we were able to obtain concentration-response curves for sustained quisqualate receptor responses. The correlation is not as strong as that for the similar comparison for NMDA receptors, but quisqualate receptor binding K_ds and EC_{50}s determined in voltage clamp experiments exhibit a constant relationship over a 3 log-unit range of agonist potency (Fig. 9). However, the voltage clamp data suggest EC_{50}s are on average 270-fold lower in affinity than that obtained by inhibition of ^{3}H-AMPA binding. The potency sequence for sustained responses at quisqualate receptors followed the order quisqualate > AMPA > L-glutamate > *S*-sulfo-L-cysteine > L-serine-*O*-sulfate > L-homocysteate ≥ L-homocysteinesulfinate > L-cysteate > L-cysteinesulfinate (≥ indicates K_ds not significantly different, *t*-test, $p > 0.10$). This is broadly similar to the potency sequence obtained with NMDA receptors, with the exception that quisqualate and AMPA are not NMDA receptor agonists at the concentrations tested, and a plot of quisqualate receptor EC_{50} versus NMDA receptor EC_{50} shows a good correlation (Fig. 10).

Sustained responses to kainate and domoate

All the excitatory amino acids tested in this study that produce responses at non-NMDA receptors activate a rapidly desensitizing response, similar to that produced by AMPA and by quisqualate. We have also examined some other amino acids that do not occur in the brain, including D-cysteate, willardine,

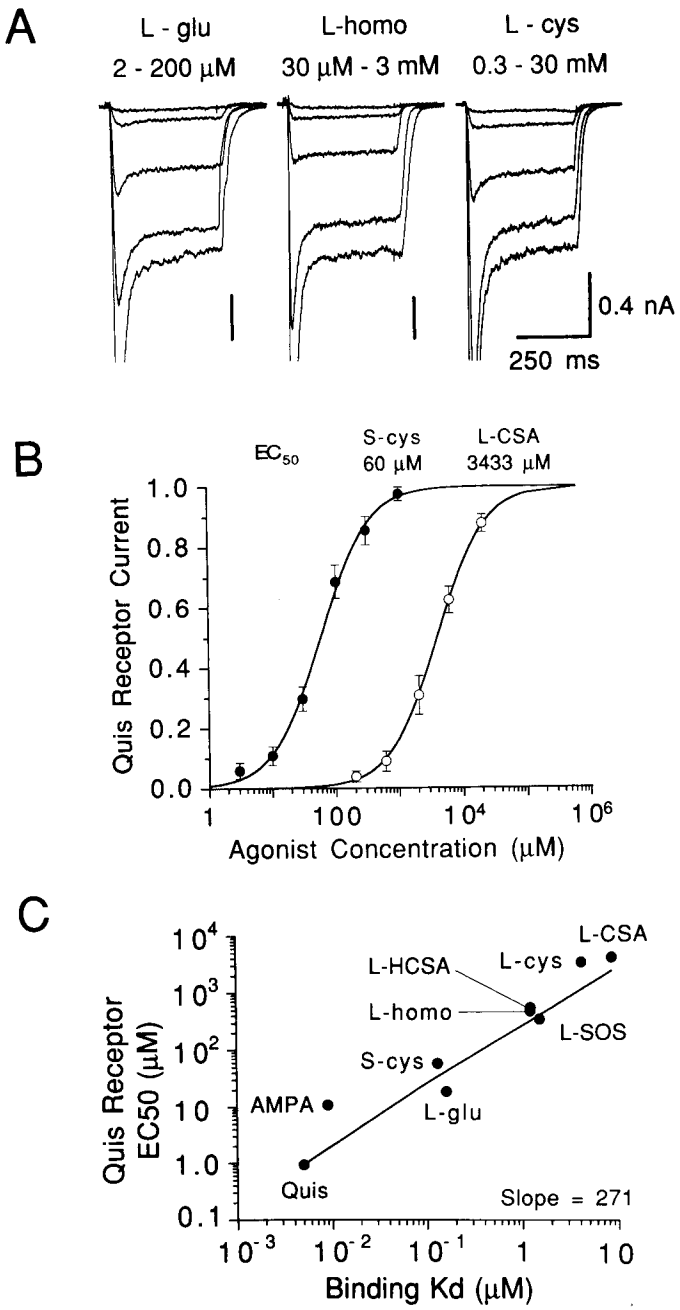

Figure 9. Dose-response curves for sustained responses at quisqualate receptors. *A*, Responses to L-glutamate, L-homocysteate, and L-cysteate, at the concentrations indicated above each record. Each agonist was applied to a different cell, and the response scaled to produce comparable peak amplitudes for illustration. The rapidly desensitizing response at quisqualate receptors is clipped at the gain used for display. *B*, Dose-response curves of similar responses for *S*-sulfo-L-cysteine and L-cysteinesulfinate recorded in individual neurons were normalized with respect to the maximum current (I_{max}) derived from analysis using the logistic equation. The results of this analysis were pooled for illustrative purposes; each data point is the mean ± SD of 9–12 observations from 5–6 cells. The concentration calculated for 50% of maximal activation (EC_{50}) and indicated above each dose-response curve was obtained by analysis of the pooled data. *C*, EC_{50}s estimated for sustained responses at quisqualate receptors, using the logistic equation, are plotted versus K_ds estimated by Murphy and Williams (1987), using displacement of ^{3}H-AMPA. The fit is by linear regression, weighting data points inversely with their standard deviation.

Table 2. Quisqualate receptor structure-activity relationships[a]

Agonist	Logistic equation[b] EC_{50} (μM)	Logistic equation[b] n	2-site model[b] K_d (μM)	Binding[c] K_d (μM)
Quisqualate	0.9 ± 0.05	1.5 ± 0.16	0.5 ± 0.1	0.005
AMPA	11 ± 1.5	1.6 ± 0.18	5 ± 0.6	0.009
L-Glutamate	19 ± 5.1	1.5 ± 0.16	9 ± 2.9	0.16
S-sulfo-L-cysteine	59 ± 7.8	1.2 ± 0.25	24 ± 3.1	0.13
L-Serine-*O*-sulfate	348 ± 85	1.3 ± 0.17	153 ± 42	1.5
L-Homocysteate	477 ± 139	1.4 ± 0.07	216 ± 60	1.2
L-HCSA	546 ± 91	1.2 ± 0.23	220 ± 44	1.2
L-Cysteate	3310 ± 547	1.2 ± 0.12	1388 ± 270	4.1
L-CSA	3981 ± 741	1.2 ± 0.17	1673 ± 421	8.5

Quisqualate receptor sustained current response EC_{50}s and Hill coefficients (n), obtained using the logistic equation; microscopic K_ds, obtained using a 2-site model, with independent binding (equation 1); K_ds estimated using displacement of ^{3}H-AMPA. Values are the means from analysis of dose-response curves recorded in individual neurons.

[a] Responses measured at equilibrium, following decay of rapidly desensitizing current; EC_{50}s for the transient current are approximately 10-fold lower affinity.

[b] Values are mean ± SD of 7–14 observations from 4 to 7 cells per agonist.

[c] Binding data from Murphy and Williams (1987) for displacement of ^{3}H-AMPA.

bromowillardine, and chlorowillardine; all produced rapidly desensitizing responses. To date we have found only 2 amino acids that activate large-amplitude, nondesensitizing responses at non-NMDA receptors: kainate and domoate (Fig. 11).

Concentration-response curves for kainate and domoate were well fit by the logistic equation (Fig. 11) and revealed domoate to be approximately 10-fold more potent than kainate. EC_{50}s and Hill coefficients were: kainate, 143 ± 17 μM, n = 1.36 ± 0.12 (8 observations on 5 cells); domoate, 13.2 ± 5.3 μM, n = 1.23 ± 0.08 (6 observations on 4 cells). The value for kainate is similar to that reported by Priestley et al. (1989) in experiments on embryonic rat cultured cortical neurons (EC_{50} = 144 μM, n = 1.56), but is of lower affinity than that estimated by Verdoorn and Dingledine (1988) from experiments on messenger RNA injected oocytes (kainate EC_{50} = 98 μM, n = 1.58), although, in this preparation, results with domoate (EC_{50} = 14 μM, n = 1.47) are similar to those reported here. As described previously for NMDA and quisqualate receptor responses, dose-response curves for kainate and domoate were also well fitted by a 2-site model with independent binding sites, with microscopic K_ds of 62 ± 6 μM for kainate and 5.4 ± 2.1 μM for domoate. The high potency of domoate is of particular interest in view of the neurotoxic action of this compound in humans following ingestion of domoate-contaminated bivalves (Glavin et al., 1989).

Discussion

Utilizing the flow pipe technique developed by Yellen (1982) and improved by Johnson and Ascher (1986), we have measured amino acid concentrations required for activation of NMDA and non-NMDA receptors on mouse cultured hippocampal neurons. For both NMDA and quisqualate receptors there was a wide range in agonist potency for amino acids considered to have physiological or pathophysiological roles in the central nervous system (1000-fold for NMDA receptors, and >100-fold for quisqualate receptors). For all endogenous agonists, the concentrations of amino acid required for activation of quisqualate receptors were considerably higher than those required for activation of NMDA receptors; this is likely to be of great significance for understanding the behavior of those central excitatory synapses which utilize both NMDA and non-NMDA receptors (e.g., Forsythe and Westbrook, 1988; Bekkers and Stevens, 1989). The interpretation of our results in the context of the synaptic activation of glutamate receptors is discussed further in Mayer (1989). We will discuss 3 aspects of our work in more detail: (1) comparison with results obtained *in vivo* or in isolated preparations of the adult central nervous system; (2) analysis of dose-response curves; and (3) differences between NMDA and quisqualate receptors.

Comparison with previous work

Development by Watkins and Evans (1981) of the kainate, NMDA, and quisqualate receptor classification scheme for CNS glutamate receptor subtypes was based on results obtained using microiontophoretic or bath application of amino acids in experiments on intact or isolated preparations from adult cats, rats, and frogs. Their experiments showed that the endogenous amino acids L-glutamate and L-aspartate were mixed agonists with activity at both NMDA and non-NMDA receptors, but the experiments did not provide reliable quantitative information concerning their potency. Our experiments extend this scheme and show that in dissociated neurons responses to excitatory amino acids arise from 4 types of activity: glycine-sensitive responses at NMDA receptors; strongly desensitizing responses at quisqualate receptors; small-amplitude, nondesensitizing responses to quisqualate receptor agonists; large-amplitude, nondesensitizing responses to kainate and domoate (see also Trussell et al., 1988; Mayer and Vyklicky, 1989). Excitatory amino acids with mixed agonist action activate responses similar to those produced by both NMDA and quisqualate but do not produce large-amplitude sustained responses of the type exemplified by kainate. Because agonist concentration changes produced by iontophoretic or bath application of excitatory amino acids *in vivo* or in brain slice preparations would be expected to occur slowly, it is likely that the non-NMDA component of responses to mixed agonists recorded in previous experiments corresponds to the small-amplitude, sustained response at quisqualate receptors observed in our study and does not reflect the

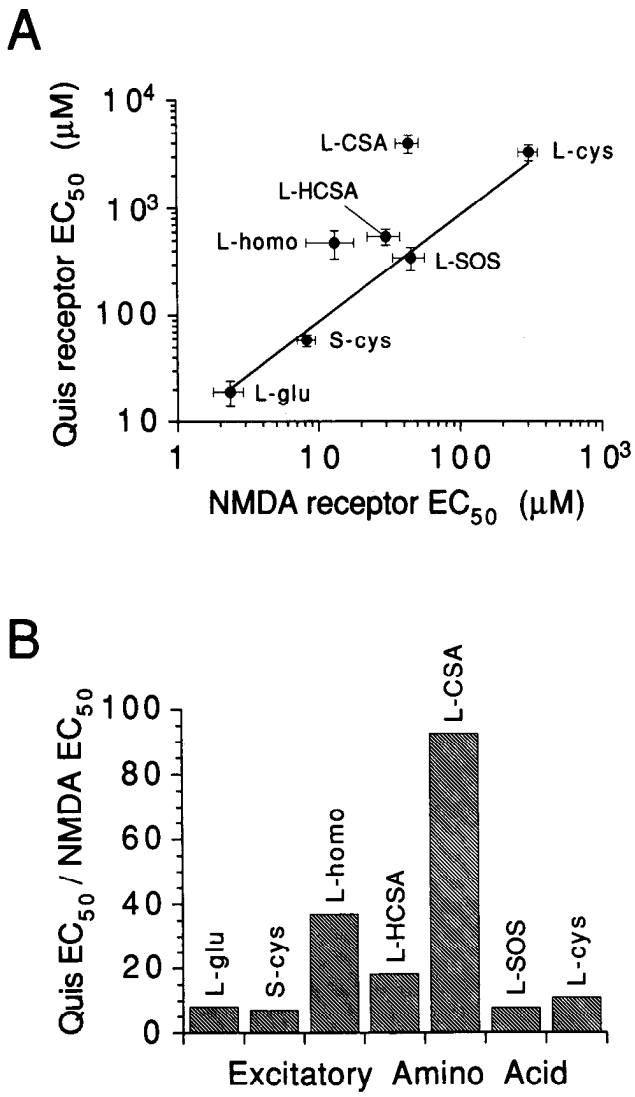

Figure 10. Comparison of agonist potency at NMDA and quisqualate receptors. *A*, EC_{50}s for activation of sustained responses at quisqualate receptors versus EC_{50}s for activation of NMDA receptors for 7 excitatory amino acids with mixed agonist activity. The potency rank order for each receptor is comparable over a wide range of agonist EC_{50}s. The line is fitted by linear regression, weighting data points inversely with their standard deviation. *B*, Bar graph of quisqualate receptor EC_{50}s divided by NMDA receptor EC_{50}s, emphasizing the relative selectivity of L-homocysteate and L-cysteinesulfinate for NMDA receptors. Quisqualate receptor EC_{50}s for the remaining 5 agonists are, on average, 10.4 times lower affinity than those for activation of NMDA receptors. Based on results with L-glutamate (see Fig. 8), EC_{50}s for activation of the rapidly desensitizing response at quisqualate receptors would be approximately 100-fold lower affinity than those for activation of NMDA receptors.

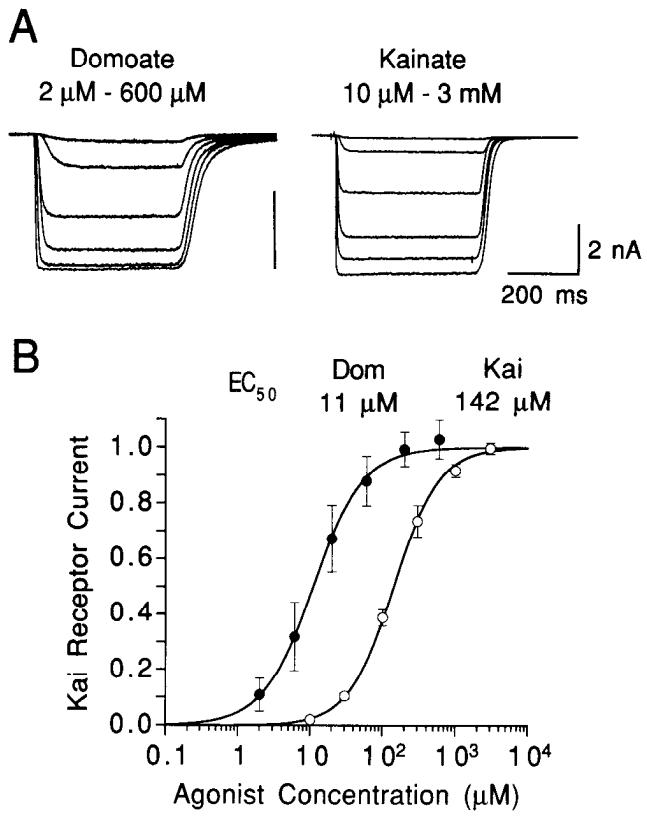

Figure 11. Nondesensitizing responses to kainate and domoate. *A*, Examples of responses to kainate and domoate at the concentrations indicated above each set of traces. Responses were recorded from 2 neurons and for display purposes are plotted to have similar maximum amplitude. *B*, Dose-response curves recorded in individual neurons were normalized with respect to the maximum current (I_{max}) derived from analysis using the logistic equation. The results of this analysis were pooled for illustrative purposes; each data point is the mean ± SD of 6–8 observations from 4–5 cells. The concentration calculated for 50% of maximal activation (EC_{50}) and indicated above each dose-response curve was obtained by analysis of the pooled data.

rapidly desensitizing response produced by rapid application of quisqualate receptor agonists.

The measurement of agonist selectivity and potency in intact preparations is distorted by at least 2 factors, which are not present in experiments on isolated cells utilizing fast perfusion techniques. In microiontophoretic experiments a nonuniform agonist concentration profile would be expected to arise as a result of release of amino acid from a point source, with high amino acid concentrations close to the iontophoretic pipette and progressively lower concentrations around nerve cell processes distal to the point of release. This effect will distort the profile of action of amino acids with activity at more than one subtype of glutamate receptor if the potency of agonist action at each receptor is not identical.

The bath application of amino acids also fails to achieve a uniform agonist concentration in intact tissue when uptake of agonist into nerve and glial cells alters the amino acid concentration profile in the extracellular space. Experiments by Garthwaite (1985) showed that the action of L-glutamate at NMDA receptors was essentially undetectable in slices of cerebellum, except at mM concentrations of amino acid, because uptake of L-glutamate hinders penetration of this amino acid into tissue slices. Experiments by Cox et al. (1977) revealed a similar uptake mechanism for L-homocysteate and block of that mechanism (most likely by competition) by L-homocysteinesulfinate, L-cysteate, and L-cysteinesulfinate. It is therefore probable that the selective action of these sulfur amino acids at NMDA receptors will also be distorted by uptake mechanisms in tissue slices and *in vivo*.

Both the ratio of affinities of excitatory amino acids for activation of NMDA versus quisqualate receptors, and the ratio of their affinities for activation of these receptors versus their affinity for uptake carriers, will be important in determining the selectivity of action of experimentally applied amino acids in

intact preparations. Our results show that the degree of mixed agonist action will be determined to a large extent by the concentration of agonist in the extracellular space around individual neurons. If high concentrations of mixed agonist must be applied to overcome the effect of uptake, then the selective activation of NMDA receptors by low concentrations of mixed agonists will not be observed. The distorting influence of the uptake of amino acids on the measurement of agonist potency *in vivo* would be expected to fall with a decrease in agonist potency, because agonists show better penetration into neural tissue at high concentrations. For example, L-cysteic acid should show greater selectivity for NMDA versus non-NMDA receptors in intact preparations than high-potency agonists such as L-glutamate, even though the ratio of the affinities of these 2 agonists for activation of quisqualate versus NMDA receptors is similar.

The results of our experiments, together with studies by Garthwaite (1985), emphasize the extreme difficulty of measuring agonist potency and selectivity for activation of glutamate receptor subtypes *in vivo,* or in brain slice or hemisected spinal cord preparations *in vitro.* Although our experiments, on embryonic mouse neurons, are unlikely to be distorted by the influence of uptake and nonuniform agonist concentration which can occur in experiments on intact preparations, they are on a different species from that studied in most other laboratories, and it is probable that, as for peripheral nicotinic (Mishina et al., 1986) and central glycinergic receptors (Hoch et al., 1989), adult and embryonic forms of glutamate receptors will exist and may have different properties. Despite this potential limitation, our results are in broad agreement with earlier work on the adult nervous system from several laboratories concerning the selectivity and relative potency of endogenous amino acids for NMDA and non-NMDA receptors and provide quantitative information not available from previous studies. Our potency sequence for NMDA receptor agonist activity closely matches that determined by measuring displacement of a radiolabelled NMDA antagonist (Olverman et al., 1988) and confirms that in the absence of uptake L-glutamate ($EC_{50} = 2.3$ μM) is the most potent NMDA receptor agonist identified to date; *S*-sulfo-L-cysteine (8.2 μM), L-homocysteate (12.9 μM), L-homocysteinesulfinate (29.9 μM), L-cysteinesulfinate (43 μM), and L-serine-*O*-sulfate (45 μM) are also potent agonists and, like L-glutamate, can in addition activate quisqualate receptors, although with lower potency.

In view of recent interest concerning a neurotransmitter role for L-homocysteate and other sulfur amino acids (Do et al., 1986), together with controversy concerning the selectivity of action of L-homocysteate at NMDA versus non-NMDA receptors (Peet et al., 1987; Herrling et al., 1989), our measurement of agonist potency for the above compounds should be helpful in considering their action in intact tissues. For 5 of the mixed agonists studied in our experiments, including L-glutamate and L-serine-*O*-sulfate, the ratio of EC_{50}s for equilibrium responses at quisqualate versus NMDA receptors ranged from 7.1 to 18.3 (mean 10.4 ± 4.62), indicating modest selectivity for NMDA receptors. In contrast, the ratio for L-homocysteate (36.9) and for L-cysteinesulfinate (92.5) indicates that these agonists have greater selectivity for NMDA than quisqualate receptors. The case of L-cysteinesulfinate is particularly striking; the EC_{50} for activation of NMDA receptors (43 ± 8 μM) is statistically indistinguishable from that of L-serine-*O*-sulfate (44.7 ± 11.4 μM), while the EC_{50}s for activation of quisqualate receptors by these agonists are strikingly different (3.98 ± 0.74 mM versus 348 ± 85 μM). Our results support the selective activation of NMDA receptors by L-homocysteic acid, but only over a narrow concentration range, because L-homocysteic acid is also a potent quisqualate receptor agonist. In contrast, L-aspartate is a completely selective NMDA receptor agonist.

Analysis of dose-response curves

Four major difficulties arose in the analysis of dose-response curves: (1) the distorting influence of desensitization, which under the conditions used for our experiments is negligible for NMDA receptors, but of considerable importance in the study of the peak response to quisqualate-like agonists; (2) a lack of information concerning rate constants for channel opening and closing for both NMDA and quisqualate receptors, which is required for full interpretation of dose-response curves fitted with models of the type developed by Colquhoun and Ogden (1988); (3) interpretation of the occurrence of agonist versus antagonist preferring states of the NMDA receptor (Monaghan et al., 1988); and (4) interpretation of the equilibrium response at quisqualate receptors.

A detailed analysis of models for receptor activation has not yet been reported for any of the glutamate receptor subtypes present in the mammalian CNS. For the peripheral nicotinic acetylcholine receptor, and for the ATP-activated receptor coupled to cation selective channels on sensory neurons, models with independent binding sites for 2 molecules of acetylcholine (Colquhoun and Ogden, 1988), or 3 molecules of ATP (Bean, 1990), give good fits to experimental dose-response curves and have the attraction that they are physically implicit concerning the number of subunits with agonist binding sites. In the case of the peripheral nicotinic receptor, biochemical and genetic techniques do actually reveal 2 subunits with binding sites for acetylcholine (e.g., Changeux et al., 1984); the structure of ATP receptors is unknown. As noted by Colquhoun and Ogden (1988), without independent kinetic data it is difficult to distinguish between independent binding site models and models in which the agonist binding sites have intrinsically different affinities or exhibit cooperative binding. The limiting slope of 2 for activation of NMDA receptors by low doses of agonist indicated that a 2-site independent subunit model was appropriate for the NMDA receptor. This is the simplest scheme that provides a close match between data from binding and voltage clamp experiments. Further experiments with kinetic analysis of the rate of activation of NMDA receptors may help to distinguish among other more complicated models.

Our analysis of NMDA receptor dose-response curves, using a 2-site model with independent binding, did not include calculation of the influence of β and α, the rate constants for channel opening and closing. Including values for β and α in the following model:

$$A + R \underset{k_{off}}{\overset{2k_{on}}{\rightleftharpoons}} AR + A \underset{2k_{off}}{\overset{k_{on}}{\rightleftharpoons}} A_2R \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{open}$$

modifies calculation of the dose-response curve from that given in Equation 1 as follows:

$$I = I_{max} \times \frac{C^2 \times \frac{\beta}{\alpha}}{1 + 2C + \left[C^2 \times \frac{\beta}{\alpha}\right]}. \quad (2)$$

For any given microscopic K_d, larger values of β/α produce leftward shifts of the agonist dose-response curve, while β/α values smaller than 1 produce rightward shifts of the dose-response curve, with a progressive reduction in slope. In 12 cases, when both β/α and the microscopic K_d were allowed to float in the dose-response equation used to fit responses to the action of L-glutamate at NMDA receptors, we obtained a value of 9.0 ± 3.1 for β/α, while the microscopic K_d increased from 1.14 ± 0.2 to 4.0 ± 0.82 μM.

For a 2-site model with $\beta/\alpha = 1$, at agonist concentrations equal to the microscopic K_d, the probability of channel opening (0.20) is quite low. As noted earlier, there is no information concerning NMDA receptor channel kinetics that allows calculation of opening and closing rate constants. But it is of interest that, to explain the kinetics of action of the open channel blocker MK801, Huettner and Bean (1988) concluded that individual NMDA receptor channels spend little time in the open state when activated by 30 μM NMDA and 1 μM glycine (Po ≈ 0.002), conditions that in our experiments produce approximately 50% of maximal activation. If this is true, then the microscopic K_d for binding of agonists to NMDA receptors would be considerably higher affinity than suggested either by our analysis or by binding studies. Also, the kinetics of activation of NMDA receptors determined by high resolution analysis of single channel data are in fact much more complicated than those recorded for peripheral nicotinic receptors (Howe et al., 1988) and would not be predicted by simple linear receptor activation schemes of the type drawn above. Further analysis of NMDA receptor function will be required to unravel this complexity.

Although analysis of voltage clamp data with a 2-site model, neglecting the influence of opening and closing rate constants, gave very good numerical correspondence between microscopic K_ds calculated from voltage clamp data and K_d values for displacement of the binding of AP5, recent experiments with radiolabeled agonists suggest a further complication. In binding experiments ligand affinity for NMDA receptors varies depending on whether labeled agonists or antagonists are used to measure receptor occupancy. As a result, the correspondence between microscopic K_d values calculated from voltage clamp data and binding K_d values would be less good if displacement of agonist binding were used for the comparison. Indeed, although K_d values obtained by displacement of agonist have not been reported for all the amino acids used in this study, results with L-glutamate, L-aspartate, and NMDA suggest that K_d values calculated using displacement of ^{3}H-L-glutamate will be 3 times higher affinity than those calculated using displacement of ^{3}H-D-AP5 (e.g., Monaghan et al., 1988; Olverman et al., 1988).

For quisqualate receptor agonists, measuring the peak response before it is reduced by the onset of desensitization is technically challenging, and it is likely that this requirement was only partially met in our experiments. Our value for the EC_{50} for L-glutamate, 480 μM, is in reasonable agreement with those of 1.1 mM, obtained by Kiskin et al. (1986), and 510 μM, obtained by Trussell and Fischbach (1989). The Hill coefficient of 1.1 estimated in our experiments agrees better with that of 1.0 estimated by Kiskin et al., versus 1.95 estimated by Trussell and Fischbach, but our estimate is likely to be lower than the true value if, as is probable in our experiments, solution changes around the entire cell were slow relative to the kinetics of desensitization.

The sustained response evoked by quisqualate receptor agonists could arise from either of the following alternative mechanisms: desensitization at quisqualate receptors could be incomplete, or the sustained response could be due to activation of an additional receptor channel complex which does not show desensitization (sustained responses to quisqualate and AMPA can be evoked for several minutes). In the latter case, it is plausible that kainate and domoate would be full agonists at this receptor, while the other amino acids we tested would act as partial agonists. The results obtained in the present series of experiments do not allow us to discriminate among the above possibilities, but it is interesting to note that in individual cells the amplitude of quisqualate receptor sustained current responses evoked by saturating doses of amino acid differs from agonist to agonist, following the sequence L-glutamate > AMPA > quisqualate (unpublished observations).

Despite the above interpretative uncertainties, it is useful to fit dose-response curves to sustained responses evoked by quisqualate receptor agonists because this allows comparison of the potency of agonist action at NMDA and quisqualate receptors. With the exception of responses to L-homocysteate, L-cysteinesulfinate, and, to a lesser extent, L-homocysteinesulfinate, the potency sequence at NMDA and quisqualate receptors is highly correlated over a 100-fold range and decreases in the order L-glutamate > *S*-sulfo-L-cysteine > L-serine-*O*-sulfate > L-cysteate. Thus the agonist recognition sites at NMDA and quisqualate receptors must share a number of similar features.

Differences between NMDA and quisqualate receptors

An important finding revealed by our experiments is the substantial difference in the potency of mixed agonists for activation of NMDA versus quisqualate receptors, with EC_{50}s for the rapidly desensitizing quisqualate receptor response for all agonists estimated to be at least 100 times higher than for NMDA receptors. The quisqualate receptor equilibrium potency sequence differs from that at NMDA receptors in 3 aspects. First, several compounds that are potent NMDA receptor agonists are inactive at quisqualate receptors (L-aspartate, NMDA, homoquinolinate). Second, quisqualate and AMPA do not produce significant activation of NMDA receptors at concentrations that produce full activation of the sustained response at quisqualate receptors. Third, sulfonic and sulfinic derivatives of glutamate are of nearly equal potency at quisqualate receptors, while at NMDA receptors the glutamate sulfonic acid analog (homocysteic acid) is 2.3 times more potent than the corresponding sulfinic acid analog. This sequence is reversed for the action of the aspartate sulfonic and sulfinic analogs at NMDA receptors, and L-cysteinesulfinate is 7 times more potent than L-cysteic acid. However, these compounds are of similar potency at quisqualate receptors.

The most striking difference between NMDA and quisqualate receptors on embryonic mouse hippocampal neurons is the complete lack of activity of L-aspartate, NMDA, and homoquinolinate at quisqualate receptors. Even at a concentration of 30 mM L-aspartate fails to activate quisqualate receptors. This is consistent with strong voltage sensitivity of the response to L-aspartate in the presence of extracellular Mg (Mayer and Westbrook, 1984) and the response of type-2 astrocytes to kainate and quisqualate but not L-aspartate and NMDA (Usowicz et al., 1989). However, previous work suggests that L-aspartate activates both NMDA and non-NMDA receptors in intact preparations (Watkins and Evans, 1981). This difference could arise from either a previously uncharacterized aspartate receptor not present in cell culture preparations of embryonic mouse spinal

cord or hippocampus, or release of L-glutamate induced by heteroexchange of L-aspartate for L-glutamate. We are unable to distinguish between these possibilities.

It is notable that the excitatory amino acids which did not activate a rapidly desensitizing response at quisqualate receptors (L-aspartate, NMDA, and homoquinolinate), or which were of very low potency (13 mM L-cysteinesulfinate, 90 mM L-cysteate, and 100 mM quinolinate produced less than one-fourth the peak response evoked by 700 μM L-glutamate), either have short chain lengths between the α- and ω-carboxyl groups or their equivalents (L-aspartate, NMDA, L-cysteinesulfinate, and L-cysteate) or are in a sterically restricted conformation which limits the maximal distance that can separate the α- and ω-carboxyl groups (quinolinate and homoquinolinate).

The most parsimonious explanation for the selective action of aspartate on NMDA receptors, and the mixed agonist action of glutamate at both NMDA and quisqualate receptors, is that the agonist recognition sites on quisqualate receptors for the α- and ω-carboxyl groups are further apart than those on NMDA receptors. Before commencing experiments with the sulfur amino acids, we predicted that the sulfonic and sulfinic analogs of aspartic acid would also be completely selective for NMDA receptors, and that homoquinolinic acid, because of the glutamate-like chain length between the ω- and α-carboxyl groups, would be a good agonist at quisqualate receptors. Molecular models provide some insight as to why these predictions were not met. The larger size of the sulfur atom in the ω-position of sulfonic and sulfinic amino acids extends the separation that is possible between electronegative oxygen atoms on the ω-sulfur and α-carboxyl groups beyond that which can be achieved in aspartic acid, where a carbon atom forms the ω-carboxyl group. As a result, cysteic acid and cysteinesulfinic acid are of intermediate chain length between glutamate and aspartate and may be able to extend sufficiently to activate quisqualate receptors. Homoquinolinate and quinolinate are conformationally restricted agonists, and although the number of carbon atoms between the α- and ω-carboxyl groups in these compounds is identical to that in glutamate and aspartate, respectively, the maximum separation possible between the α- and ω-carboxyl groups in quinolinic and homoquinolinic acids is less than is possible in aspartate and glutamate, because several of the methylene groups form part of an unsaturated ring structure. Indeed, the extremely low potency of quinolinic acid may arise because the α- and ω-carboxyl groups are too close and cannot achieve the optimal separation for activation of even NMDA receptors.

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