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Identification of Amino Acid Residues of the NR2A Subunit That Control Glutamate Potency in Recombinant NR1/NR2A NMDA Receptors

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The NMDA type of ligand-gated glutamate receptor requires the presence of both glutamate and glycine for gating. These receptors are hetero-oligomers of NR1 and NR2 subunits. Previously it was thought that the binding sites for glycine and glutamate were formed by residues on the NR1 subunit. Indeed, it has been shown that the effects of glycine are controlled by residues on the NR1 subunit, and a “Venus flytrap” model for the glycine binding site has been suggested by analogy with bacterial periplasmic amino acid binding proteins. By analysis of 10 mutant NMDA receptors, we now show that residues on the NR2A subunit control glutamate potency in recombinant NR1/NR2A receptors, without affecting glycine potency. Furthermore, we provide evidence that, at least for some mutated residues, the reduced potency of glutamate cannot be explained by alteration of gating but has to be caused primarily by impairing the binding of the agonist to the resting state of the receptor. One NR2A mutant, NR2A(T671A), had an EC50 for glutamate 1000-fold greater than wild type and a 255-fold reduced affinity for APV; yet it had single-channel openings very similar to those of wild type. Therefore we propose that the glutamate binding site is located on NR2 subunits and (taking our data together with previous work) is not on the NR1 subunit. Our data further imply that each NMDA receptor subunit possesses a binding site for an agonist (glutamate or glycine).

Key words: N-methyl-D-aspartate (NMDA); glutamate; receptor; recombinant; subunit; ligand; binding; gating; single-channel; APV; Schild plot; affinity; oocyte; mutant; kinetic

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MATERIALS AND METHODS

Expression plasmid constructs. The wild-type expression plasmids for the rat NR1 (1a splice form) and NR2A NMDA receptor subunits were as in Kuner and Schoepfer (1996). Mutations were introduced into the NR2A sequence by PCR-based mutagenesis, To facilitate cloning and identification of mutants, some NR2A mutants carried additional silent nucleotide substitutions. PCR-generated DNA segments and cloning sites were confirmed by DNA sequencing.

Heterologous expression of NMDA receptors. cRNA was synthesized as runoff transcripts from linearized plasmid DNA using the Promega (Madison WI) RiboMax RNA synthesis kit. Reactions were supplemented with 0.75 mM GTP, 50 IU/ml RNasin, 300 μg/ml actinomycin D, 50 μg/ml streptomycin (BRL, Bethesda, MD), 50 μg/ml amphotericin B (BRL, Bethesda, MD), 50 μg/ml streptomycin (BRL, Bethesda, MD), and 30 μg/ml trypomycin (BRL) and 30 μg/ml trypomycin (BRL). Tocris), followed by storage at 4°C.

Glutamate stock solutions (1 mM in H2O) were neutralized with N-methyl-D-glucamine. APV was stored as 100 mM stock neutralized with NaOH. All stock solutions were stored at -20°C. For measurements NMR was further supplemented with 30 mM glycine (for glutamate dose–response measurements), or with 30 mM to 1 mM glutamate, depending on the glutamate potency of each receptor (for glycine dose–response measurements).

Application of solutions was computer-controlled by the program EggWorks. Applications of agonist were kept as short as possible, consistent with reaching a plateau or peak response. Data were recorded simultaneously on a chart recorder and on a computer hard disk. For digital recording, data were filtered at 100 Hz digitized at 300 Hz and reduced to a final sampling rate of 10 Hz (oversampling with equal weight averaging of 30 samples) before storage.

Data analysis for dose–response curves. Dose–response curves were fitted individually for each oocyte with the Hill equation:

$$I = \frac{I_{max}}{1 + \left(\frac{EC_{50}}{[A]}\right)^n}$$

where $I_{max}$ is the Hill coefficient, $I_{max}$ is the maximum current, $[A]$ is the concentration of agonist, and $EC_{50}$ is the concentration of agonist that produces a half-maximum response. Each data point was then normalized to the fitted maximum of the dose–response curve.

The normalized values were then pooled and averaged for each mutant and fitted again with the Hill equation using the Scan program (Colquhoun and Sigworth, 1995).

Schild analysis. APV antagonism was examined by the Schild method (Arunlakshana and Schild, 1959). Recordings were done simultaneously on a chart recorder and on a computer hard disk. For digital recording, data were filtered at 100 Hz digitized at 300 Hz and reduced to a final sampling rate of 10 Hz (oversampling with equal weight averaging of 30 samples) before storage.

Data analysis for concentration jump experiments. Dose–response curves were fitted individually for each oocyte with the Hill equation:

$$I = \frac{I_{max}}{1 + \left(\frac{EC_{50}}{[A]}\right)^n}$$

where $I_{max}$ is the Hill coefficient, $I_{max}$ is the maximum current, $[A]$ is the concentration of agonist, and $EC_{50}$ is the concentration of agonist that produces a half-maximum response. Each data point was then normalized to the fitted maximum of the dose–response curve.

The normalized values were then pooled and averaged for each mutant and fitted again with the Hill equation using the Scan program (Colquhoun and Sigworth, 1995).

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RESULTS

NR2A mutations

Ten mutants containing single amino acid substitutions of the NR2A subunit were generated by site-directed mutagenesis, at positions that were chosen either because they control glycine potency on NR1 subunits, or because they control amino acid binding in bacterial binding proteins. Alignment of NR2 sequences with bacterial amino acid binding proteins (Fig. 1A) reveals limited linear sequence identity in segments N terminal of M1 and C terminal of M3, in extracellular domains referred to as S1 and S2, respectively (Stern-Bach et al., 1994). The corresponding segments on NR1 harbor residues that control glycine potency (Kuryatov et al., 1994). Mutants were generated according to the following rule: uncharged amino acids were mutated into alanine, positive charges were substituted by negative charges, and in addition aromatic residues were replaced by another aromatic residue.

Recombinant coexpression of NR1 cRNA with any of the 10 mutant NR2A cRNAs resulted in robust inward currents after the application of glutamate in the presence of glycine (Fig. 1B). Injection of similar amounts of cRNA resulted in maximal agonist-induced currents of comparable magnitude; in particular, $I_{max}$ for the mutants was generally not lower than $I_{max}$ for the wild type (the values are given in the legend of Fig. 2).

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Reduced potency of glutamate

Dose–response curves were recorded (Fig. 1B) in conditions (180 μM Ba²⁺) designed to minimize the artifacts caused by agonist-induced influx of divalent cations (Leonard and Kelso, 1990; Williams, 1993). With the exception of NR2A(G664A), all of the mutations we tested shifted the dose–response curves for glutamate to the right (Fig. 2A). For all mutations except S670A (and G664A), the potency of glutamate was reduced by more than fivefold. Three mutations (H466A, G669A, and T671A) decreased the potency of glutamate by more than two orders of magnitude (Table 1). The fitted dose–response curves (Fig. 2A) show that the Hill coefficients for most of the 10 mutant receptors

Figure 1. Single amino acid substitutions in the NR2A subunit generated functional NR1/NR2A receptors. A, Amino acid sequence alignment of rat NR1 (Moriyoshi et al., 1991) and NR2 subunits (Monyer et al., 1992; Ishii et al., 1993) with the bacterial periplasmic binding proteins LAOBP (Oh et al., 1993) and HISJ (Oh et al., 1994). Residues that reduced glycine potency when mutated in NR1 are marked by an arrow above the sequence alignment. NR2A residues that were mutated in this study are marked below the sequence alignments by an arrow if the mutation reduced potency by more than a factor of 2; otherwise they are indicated by a minus sign. Numbers indicate the first residue of the alignment; numbering is for mature proteins without signal peptides. B, Glutamate-activated currents recorded in the two-electrode voltage-clamp configuration from an oocyte expressing wild-type receptors (left) and an oocyte expressing T671A mutant receptors (right). Increasing concentrations of glutamate were applied in the presence of 30 μM glycine.
are not much different from the value found for wild-type NR1/NR2A receptors. The slopes for H466F and V666A are probably lower, and for K465E may be higher, than for the wild type, but the three mutations with the largest increase in EC$_{50}$ show little or no change of the Hill slope.

In contrast to the considerable effect of the mutations on glutamate potency, the potency of the coagonist glycine was barely affected; the EC$_{50}$ values for all 10 mutants lay within a factor of 1.7 of that of the wild type, as shown in Figure 2B.

Table 1. EC$_{50}$ and Hill values for glutamate and glycine

| Glutamate | EC$_{50}$ (μM) | $n_H$  | $n$  | Glycine | EC$_{50}$ (μM) | $n_H$  | $n$  |
|-----------|----------------|--------|------|---------|----------------|--------|------|
| NR1/NR2A  | 2.89 ± 0.12    | 1.49 ± 0.09 | 8   | 2.62 ± 0.19    | 1.54 ± 0.15 | 6   |
| NR1/NR2A(N463A) | 17.9 ± 1.4    | 1.70 ± 0.18 | 3   | 2.50 ± 0.16    | 1.78 ± 0.18 | 4   |
| NR1/NR2A(K465E) | 29.3 ± 1.4    | 1.91 ± 0.18 | 4   | 3.11 ± 0.27    | 1.64 ± 0.20 | 3   |
| NR1/NR2A(H466A) | 62.4 ± 32     | 1.55 ± 0.09 | 6   | 3.67 ± 0.22    | 1.54 ± 0.12 | 4   |
| NR1/NR2A(H466F) | 38.4 ± 4.6    | 1.09 ± 0.10 | 4   | 2.10 ± 0.22    | 1.69 ± 0.24 | 3   |
| NR1/NR2A(G664A) | 2.08 ± 0.25   | 1.46 ± 0.21 | 3   | 1.69 ± 0.21    | 1.44 ± 0.21 | 3   |
| NR1/NR2A(T665A) | 19.0 ± 2.0    | 1.18 ± 0.11 | 3   | 3.86 ± 0.39    | 1.39 ± 0.15 | 5   |
| NR1/NR2A(V666A) | 33.4 ± 1.6    | 1.12 ± 0.05 | 5   | 4.53 ± 0.31    | 1.35 ± 0.10 | 4   |
| NR1/NR2A(G669A) | 927 ± 46      | 1.38 ± 0.07 | 4   | 3.58 ± 0.23    | 1.39 ± 0.10 | 4   |
| NR1/NR2A(S670A) | 4.97 ± 0.30   | 1.35 ± 0.09 | 5   | 3.49 ± 0.39    | 1.57 ± 0.24 | 3   |
| NR1/NR2A(T671A) | 2967 ± 279    | 1.30 ± 0.09 | 5   | 2.94 ± 0.23    | 1.29 ± 0.10 | 4   |
Reduced affinity for APV

To examine the underlying mechanism that is responsible for the decreased potency of glutamate (see Discussion), we analyzed the NR1/NR2A(T671A) mutant in greater detail. Schild's method (Arunlakshana and Schild, 1959) was used to determine the equilibrium constant, \( K_H \), for the competitive antagonist APV (Fig. 3).

The slope of the Schild plot was 1.06 ± 0.04 for the wild type and 0.94 ± 0.16 for T671A. These values are close to (and do not differ significantly from) 1.0, so there is no evidence against the view that APV is a competitive antagonist on both receptors. Therefore the plots were refitted with the Schild equation (slope fixed at 1), as described in Materials and Methods, and these fits are shown in Figure 3C. For the wild type we found that \( K_H = 1.26 ± 0.07 \mu M \), which is similar to the value of 1.35 \( \mu M \) reported by Verdoorn et al. (1989) for rat brain mRNA expressed in oocytes. For the T671A mutant, however, we found a much reduced affinity for APV, with \( K_H = 321 ± 30 \mu M \). This 255-fold reduction in affinity is comparable with the 1000-fold reduction of potency for glutamate.

Single-channel conductance

The single-channel currents evoked by glutamate in NR1/NR2A(T671A) were remarkably similar to those evoked in the wild type (Fig. 4). The mean chord conductances of their main level and sublevel were estimated as 51.1 ± 1.1 \( \mu S \) and 38.6 ± 0.9 \( \mu S \) \((n = 5)\), compared with values of 50.1 ± 1.4 \( \mu S \) and 38.3 ± 1.3 \( \mu S \) reported for wild-type NR1/NR2A by Stern et al. (1992). The relative areas for these two components, 85% and 15%, were also similar to those for wild type (80% and 20%). In addition, the frequencies of direct transitions from shut to main level, from shut to sublevel, and from main to sublevel of T671A channels (Table 2) were similar to those reported for the wild type by Stern et al. (1992). Like the wild-type NR1/NR2A, they showed no sign at all of the sort of temporal asymmetry shown by NR1/NR2D receptors (Wyllie et al., 1996).

Single-channel kinetics

In contrast to the close similarity of the conductances, there were kinetic differences between T671A and the wild type. For example, both mutant and wild-type open period distributions contained three exponential components (Fig. 5). The mean time constants for each component were 66.2 ± 17.0 \( \mu \text{s} \), 1.10 ± 0.34 \( \mu \text{s} \), and 0.91 ± 2.45 \( \mu \text{s} \) for T671A channels \((n = 5)\), and 47.6 ± 9.3 \( \mu \text{s} \), 0.823 ± 0.375 \( \mu \text{s} \), and 3.08 ± 0.57 \( \mu \text{s} \) for wild-type channels \((n = 5)\) (P. Béhé and D. Wyllie, personal communication). The overall mean apparent length of the open periods was 4.33 ± 0.71 \( \mu \text{s} \) for T671A compared with 1.69 ± 0.16 \( \mu \text{s} \) for the wild type (also see Fig. 4). These values are clearly different \((p = 0.0045\) by two-tailed randomization test). For this comparison, both wild-type and mutant data were analyzed with the same resolution (70 \( \mu \text{s} \) for both open and shut times). Such a resolution would mean that a substantial number of brief shuttings were missed; however, it is not possible to do a satisfactory correction for missed events without knowledge of a realistic kinetic mechanism (Colquhoun and Hawkes, 1995).

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**Figure 3.** Schild analysis of the competitive antagonism of the glutamate binding site by APV. **A.** Partial, low-concentration, glutamate dose–response curves for one oocyte expressing wild-type receptors in the absence (open squares) and presence of 3 \( \mu M \) (filled diamonds), 10 \( \mu M \) (open pentagons), 30 \( \mu M \) (filled hexagons), and 100 \( \mu M \) (open circles) APV. **B.** Partial, low-concentration, glutamate dose–response curves for one oocyte expressing T671A mutant receptors in the absence (filled pentagons) and presence of 300 \( \mu M \) (open diamonds) and 1 \( \mu M \) (filled triangles) APV. Data were fitted as in **A.** C. Schild plot for antagonism of wild-type (filled circles) and T671A (filled squares) receptors by APV using dose ratios estimated from results such as those in **A** and **B**. The points represent the means of five dose ratios. The dashed lines are free fits with slopes that are not exactly 1; the solid lines are fits of the Schild equation (both have slope = 1). Fitted lines have been extrapolated for display purposes.
Kinetic differences between mutant and wild-type receptors were also observed in concentration jump experiments. Preliminary data, shown in Figure 6, indicate that deactivation of T671A receptors, after a 100 msec jump of 10 mM glutamate, occurs with a time constant of 18.2 ± 3.0 msec (n = 4). This is approximately one order of magnitude faster than that found for the deactivation of wild-type NR1/NR2A channels after “long” pulses of glutamate (Monyer et al., 1992; Medina et al., 1995) (D. Wyllie and P. Béhé, personal communication).

**DISCUSSION**

**Glutamate effects are mediated through NR2 subunits**

We have shown that mutagenesis of NR2A residues reduced the potency of glutamate by up to 1000-fold, with very little effect on

**Table 2. Transitions between conductance levels for NR1/NR2A(T671A) channels**

| Transition type          | n (N = 10897) | %   |
|--------------------------|--------------|-----|
| Shut-sublevel            | 400          | 3.67|
| Sublevel-shut            | 366          | 3.36|
| Shut-main level          | 4210         | 38.63|
| Main level-shut          | 4241         | 38.92|
| Sublevel-main level      | 860          | 7.89|
| Main level-shut          | 820          | 7.53|

Experiments were performed in the presence of 20 μM glycine and between 60 and 200 μM glutamate.

*N* refers to the total number of transitions, longer than 2.5 *t*, analyzed from five recordings.
the potency of glycine. Our evidence strongly suggests (see below) that the binding site for glutamate on NMDA NR1/NR2A receptors is formed by NR2A subunits. The residues we found to be important in NR2A are fully conserved within the NR2 family (Fig. 1A), which suggests that in all types of NR1/NR2 NMDA receptors glutamate effects are mediated by the NR2 subunit. Comparison of our data with the results of Kuryatov et al. (1994) and Wafford et al. (1995) on glycine potency of NR1 mutations suggests that the glutamate effects mediated through the NR2 subunits, and glycine effects mediated through the NR1 subunit, are each based on the same structural design.

While the experiments described here were in progress, Laube et al. (1997) reported that mutation of NR2B residues reduced glutamate potency on NR1/NR2B receptors. Three of their mutations were equivalent to ours; for two of them we both found a comparable (approximately 10-fold) reduction in potency of glutamate (H466F, V666A), whereas for one (K465E, equivalent to NR2B K459E) we found a considerably smaller effect. For NR2A S670, we analyzed the effect of the mutation to alanine, S670A, and found only a minimal effect; the same serine to alanine mutation at the equivalent position on the NR1 subunit also has little effect on glycine potency (Kuryatov et al., 1994). In contrast, Laube et al. (1997) report a 180-fold reduction of glutamate potency for a mutation into glycine at the homologous position (S664G). The mutations that gave the three largest increases in glutamate EC\(_{50}\) in Laube et al. (1997) (236-, 180-, and 118-fold) were all at different positions from those that gave the largest shifts here (1027-, 321-, and 216-fold). Furthermore their mutant with the largest shift actually showed a decreased IC\(_{50}\) for APV, although that with the 180-fold shift showed a 40-fold increase in IC\(_{50}\). Our most-shifted mutant, T671A, showed a 255-fold increase in the equilibrium binding constant of APV. Laube et al. (1997) proposed an appealing three-dimensional arrangement for the structure of the glutamate binding site, and it will be interesting to see how this proposal can accommodate the rather different residues that seem, from the mutants tested here, to be important for glutamate binding.

Evidence that reduced potency is caused by impaired binding of glutamate

The basis for the inference that the mutated residues form part of the glutamate binding site requires clarification. A reduction of potency is not necessarily caused by a change in the binding site but can also be caused by a change of gating. In other words, the glutamate binding site might not be affected at all; furthermore, a binding assay for glutamate would not resolve this ambiguity (see Colquhoun and Farrant, 1993).

To infer that the mutated residues form part of the binding site it is necessary to show that the mutation has altered the microscopic equilibrium constant for the initial binding step: the binding of glutamate to the resting state of the receptor. If this were the only change we could say that it was very likely that these domains form part of the glutamate binding site (we do not distinguish in this paper between residues that interact directly with the ligand and residues that shape the binding site without direct interaction with the ligand).

One way to examine this problem is to use a competitive antagonist rather than an agonist. This removes most of the complications in interpretation of the effect of a mutation, because a competitive antagonist, unlike an agonist, is not expected to cause a change of the receptor conformation to higher-affinity active states (i.e., gating). Thus any change in their binding is likely to result directly from a change in the binding site. The equilibrium constant for antagonist binding, K\(_{a}\), cannot be determined from IC\(_{50}\) measurements, so Schild's method was used. It is possible that binding of the agonist might be altered in a mutant receptor without alteration of binding of a competitive antagonist, because the binding sites for the two compounds, although presumably overlapping, are not identical. However, we found
that the T671A mutation increased the equilibrium binding constant 255-fold for APV compared with wild type. Therefore in this mutant, APV binding is impaired greatly, which implies that its binding site, and therefore probably that for glutamate, has been altered.

The equilibrium concentration–response curves also suggest a primary effect of the mutation on the binding site for glutamate. The main finding is that mutations in the NR2 subunit can produce an enormous reduction in the potency of glutamate \( (EC_{50}) \), with little change in the Hill slope, and very little change in the potency of glycine (Table 1). Of the three most effective mutations, one precedes M1 (S1 domain) and the others follow M3 (S2 domain), which suggests that both regions contribute to the binding site.

A reduction in the open-shut equilibrium constant could produce this result without any detectable change in maximum response, provided that its reduced value is still such that most channels are open at equilibrium. How can we exclude this possibility? The first reason is that for many cooperative mechanisms we expect the shift of the \( EC_{50} \) to be proportional to the binding constants but roughly proportional (in the case of two bindings being required) to the square root of the gating constant (as long as this equilibrium is well over toward the open state). In this case the 1000-fold increase in \( EC_{50} \) seen with T671A would require a roughly millionfold reduction in the gating constant. If this were to occur without a drastic reduction of the maximum response, the gating constant for the wild-type channel would have to be enormously high. This does not appear to be the case, because glutamate actually behaves like a partial agonist on NMDA receptors with a maximum \( P_{\text{open}} \) of ~0.3 (Gibb and Colquhoun, 1992; Dzubay and Jahr, 1996). Therefore, such a reduction in the gating constant would cause an enormous reduction in the maximum response. In common with almost every mutation study, we have no good evidence as to whether the maximum response is changed, because of uncertainties about the relative efficiency of expression of wild-type and mutant RNAs; however, there was no systematic difference between the maxima observed (Fig. 2 legend), so it is clear that changes by a factor of one million have not occurred.

Furthermore, most kinetic mechanisms predict that the Hill slope would be reduced substantially if there were such a large effect on the gating constant as would be required to account for our observations; we observed no such reduced Hill slope.

**Interpretation of kinetic data of the T671A mutant**

In principle, effects on channel gating can be measured from kinetic analysis of single-channel recordings (Colquhoun and Sakmann, 1985). We show that transition frequencies between the conductance levels of T671A (as well as the levels themselves) are similar to those of the wild type. Therefore, at least this aspect of gating is not altered in the mutant with the largest reduction in potency.

The observation that the mean apparent open period is longer for the T671A mutant could mean that the channels stay open rather longer, which would imply some effect on the shutting rate of the channel, i.e., on the gating process. Such a change, however, is in the wrong direction to explain reduced glutamate potency. In any case it is quite conceivable that there is no real increase in the length of individual openings; the apparent increase could well result from more rapid dissociation of the agonist, which would be expected to reduce the duration and frequency of brief shuttings such that fewer of them are detected and openings appear, incorrectly, to be longer.
Figure 6 shows that the decay of the response, after rapid removal of glutamate, is roughly 10-fold faster for T671A than for wild-type receptors. The decay rate of the current is a reflection of the length of the underlying channel activation, or burst (Colquhoun et al., 1997). We have found that the individual openings, if anything, are longer in the mutant receptor, so the fast decay cannot be explained by shorter open times. The length of an activation will depend on the number of times that the channel reopens while agonist is still bound. The more rapid decay is therefore likely to result from there being fewer openings during an activation for the mutant channel compared with the wild type. This could result from either (1) an increased rate of dissociation of glutamate or (2) a reduced opening rate constant for the mutant channel. If we are right in assuming that wild-type and mutant receptors express with comparable efficiency, then, as discussed above, it is unlikely that the opening rate constant is greatly reduced in the mutant, so this is also unlikely to be the main reason for the faster decay (although a contribution from this source cannot be ruled out). Thus the main reason is likely to be that glutamate dissociates more rapidly, thereby reducing the probability that the channel will reopen. An increase in dissociation rate is consistent with a reduced affinity for glutamate.

Implications

In the absence of any completely satisfactory kinetic mechanism for the NMDA receptor, it is not possible to infer from the results, in a rigorous manner, numerical values for the microscopic equilibrium constant for the initial binding step or for the gating constants. As discussed above, it is most likely that the primary effect, at least for the three most effective mutations, is on glutamate binding.

The model of a conserved structural design of agonist binding sites of the extended ionotropic glutamate receptor family is further supported by other studies. Paas et al. (1996) investigated ligand binding to chick cerebellar kainate binding protein, and Kuusinen et al. (1995) found ligand binding by a recombinant fusion protein consisting only of fused S1 and S2 domains of an AMPA receptor.

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