0026-895X/95/020374-07$03.00/0

MOLECULAR PHARMACOLOGY, 47:374-380, 1995.

Identification of Amino Acids in the *N*-Methyl-D-aspartate Receptor NR1 Subunit that Contribute to the Glycine Binding Site

K. A. WAFFORD, M. KATHORIA, C. J. BAIN, G. MARSHALL, B. LE BOURDELLÈS, J. A. KEMP,[1] and P. J. WHITING

Neuroscience Research Centre, Merck, Sharp & Dohme Research Laboratories, Harlow, Essex CM20 2QR, UK

Received July 29, 1994; Accepted November 18, 1994

SUMMARY

The mammalian *N*-methyl-D-aspartate (NMDA) receptor complex is thought to consist of an NR1 subunit in combination with one or more of the four NR2 subunits (A, B, C, and D). When corresponding cDNAs are expressed in *Xenopus* oocytes, ion channels with the characteristic profile of NMDA receptors are formed. The receptor is unique in requiring two coagonists, glutamate and glycine, for activation of the channel. We have used site-directed mutagenesis to study amino acids in the human NR1 subunit that contribute to the glycine binding site of the NMDA receptor without affecting the agonist site for glutamate. Mutations to D481 and K483 produced receptors with up to 160-fold lower affinities for glycine, as well as other agonists and partial agonists, without affecting maximum current size or the degree of agonist efficacy. The D481A mutation also led to 40–50-fold lower affinities for two structurally diverse glycine site antagonists. From these data we propose that the carboxyl group of this aspartate interacts with the amino moiety of glycine and the equivalent group contained in other agonists and antagonists.

The NMDA receptor is found throughout the central nervous system and plays a pivotal role in neuronal processing. This ligand-gated cation channel is characteristically permeable to calcium and is voltage-dependently regulated by magnesium. The receptor is also unique in requiring two coagonists, glutamate and glycine, for activation of the channel (1). The coincidental binding of glutamate and glycine at separate sites on the NMDA receptor leads to the opening of a cation channel that is highly permeable to calcium. The discovery of the glycine binding site on the NMDA receptor has generated considerable interest in it as a therapeutic target (2, 3), and the identification of cDNAs encoding receptor subunits allows a more detailed analysis of agonist/receptor interactions. To date, two types of subunits have been cloned that can combine to form NMDA-like channels. The NR1 subunit expressed together with any of the four NR2 subunits (A, B, C, or D) results in the formation of ion channels with the characteristic profile of NMDA receptors (4–11). Although the NR2 subunits in combination with NR1 dramatically increase the amplitude of NMDA currents and do affect glycine affinity, unlike the NR1 subunits they do not produce channels gated by glutamate and glycine when expressed alone (4–9). Indeed, NR1 expressed alone in transfected cells forms a high affinity glycine binding site detected by radioligand binding (12, 13) but does not form ion channels that are detectable using whole-cell voltage-clamp recordings. This suggests that agonist binding domains are located at least on the NR1 subunit and that this site is modulated by the presence of an NR2 subunit. This hypothesis is also supported by recent evidence using site-directed mutagenesis of the NR1 subunit. Kuryatov *et al.* (14) identified aromatic residues at three positions in the amino-terminal part of the NR1 subunit, mutation of which yielded large reductions in glycine and 7-chlorokynurenic acid affinity (when the mutants were expressed together with the NR2B subunit). Those authors also noted that changes at D481 and K483 (the key residues identified in this study) resulted in a reduction in glycine affinity, but they did not investigate those mutations in any further detail.

Amino acid residues amino-terminal to putative transmembrane domain 1 of the nicotinic receptor (15), strychnine-sensitive glycine receptor (16), $GABA_A$ receptor (17), and non-NMDA receptors (18) have been proposed to contribute to agonist interactions with these ligand-gated ion channels. With this in mind, we performed a site-directed mutagenesis study to examine in detail the residues of the NR1 subunit that contribute to the glycine binding site of the NMDA receptor.

[1] Present address: Central Nervous System Department, Pharma Division Preclinical Research, Hoffmann-La Roche Ltd., CH-4002, Basel, Switzerland.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; ACBC, 1-amino-1-carboxycyclobutane; GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR1, glutamate receptor type 1.

Materials and Methods

Mutagenesis. The human NR1 and NR2A cDNAs were obtained from a human hippocampal cDNA library (19), with NR1 representing the NR1a form of the subunit (11). cDNAs were subcloned into pCDNAI/Amp (NR1a) and pCDM8 (NR2A) vectors for expression in oocytes. These eukaryotic expression vectors contain the same eukaryotic transcription cassette. Site-directed mutagenesis was carried out as described previously (20) and was confirmed by DNA sequencing.

Expression in *Xenopus* oocytes. Methods for oocyte preparation were as described previously (21). NMDA receptor cDNAs were prepared in injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, pH 7.0; filtered through nitrocellulose membranes) and mixed in a 1:3 ratio of NR1a (3 ng/μl) to NR2A (9 ng/μl). Approximately 10 nl of these cDNA mixtures were directly injected into the oocyte nucleus via a Hamilton 10-μl syringe, and the oocytes were maintained at 20° in modified Barth's medium [88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(N0_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, pH 7.5] supplemented with 10,000 units/liter penicillin, 10 mg/l streptomycin, 50 mg/l gentamycin, and 2 mM sodium pyruvate. After incubation for 2–3 days, oocytes were placed in a 50-μl bath and perfused with barium Ringers solution (115 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.8 mM $BaCl_2$, pH 7.2) at 5–10 ml/min. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and were voltage-clamped between −40 and −80 mV using an Axoclamp 2A. Agonists were applied by bath perfusion until a peak response was observed. Concentration-response curves were calculated by using a nonlinear, least-squares, fitting program with the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^n]$, where x is the drug concentration, EC_{50} is the drug concentration eliciting a half-maximal response, and n is the Hill coefficient. EC_{50} values are based on mean values calculated from curve fits for individual cells. L-689,560 and L-687,414 were synthesized at Merck, Sharp & Dohme Research Laboratories (Harlow, UK), ACBC was obtained from Tocris Neuramin, and all other compounds were obtained from Sigma or FSA Supplies.

Results

Amino acids in the amino-terminal putative extracellular domain determine glycine affinity. By mutating residues in the amino-terminal region of the human NR1a subunit (19, 22, 23), we have located amino acids involved in receptor activation by glycine. Fig. 1 shows part of the human NR1 deduced amino acid sequence (19, 22, 23), aligned with that of human NR2A (19) and two other human non-NMDA glutamate subunits, GluR1 (24) and EAA1 (25). The positions of the amino acids mutated in this study are indicated and the numbering is according to the system of Le Bourdelles *et al.*

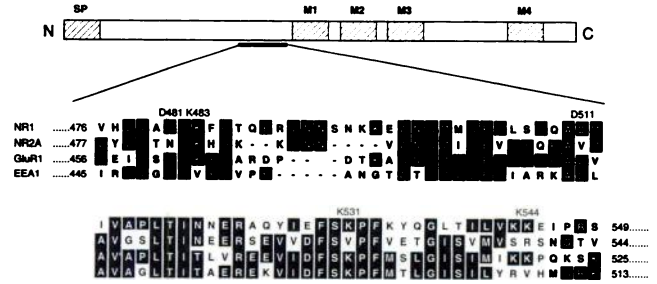


Fig. 1. Aligned sequences of part of the amino-terminal region of the human NR1 subunit (17) and the human NR2A (17), GluR1 (α1) (20), and EAA1 (kainate receptor subunit) (21) subunits, indicating the positions of amino acids that were mutated in this study. *SP*, putative signal peptide; *M1–M4*, putative transmembrane domains 1–4. Boxed regions show amino acid identity between sequences and dashes indicate gaps inserted to allow sequence alignment. The numbering of amino acids was determined by designating the initiating methionine as 1.

(19). Initially, conservative substitutions were made, because receptors that lacked activity would not enable us to draw any conclusions regarding agonist activation. The resulting NR1 mutants were coexpressed with the human NR2A subunit (19) in *Xenopus* oocytes, and concentration-response curves for glutamate and glycine were generated in the presence of a maximum concentration of the coagonist. Changes at three amino acids were found to affect the affinity for glycine, compared with the wild-type receptor. K544Q, D481N, and K483Q produced receptors with glycine affinities 5-fold (11.5 ± 1.9 μM), 7-fold (16.3 ± 2.3 μM), and 126-fold (293 ± 78 μM) lower, respectively, than the wild-type receptor affinity (2.33 ± 0.18 μM) (Table 1). The glycine affinity of an NR1 mutant containing both D481N and K483Q was even further reduced to 1548 ± 591 μM (Table 1). The affinities for glutamate of K554Q, D481N, K483Q, and the double-mutant D481N/K483Q were only slightly (2–3-fold) lower than that of the wild-type receptor (Table 1), indicating that these amino acids do not participate in the glutamate binding site. Maximum currents produced by activation of the receptor were similar for all mutants tested, with the exception of R523L, which resulted in the lack of any current in response to glutamate and glycine. Interestingly, mutation of the equivalent amino acid in the AMPA GluR1 (α1) receptor also resulted in the abolition of currents (18), suggesting that this residue may be critical for the formation of functional receptors. Mutations of a number of other amino acids in this region (Table 1) had little effect on either the glycine or glutamate EC_{50}. Others have reported that mutation of cysteine residues in this region of the NR1 subunit also has no effect (26). Fig. 2 shows membrane currents (Fig. 2a) and concentration-response curves for glycine (Fig. 2b, *top*) and glutamate (Fig. 2b, *bottom*) with wild-type NR1a/NR2A receptors and receptors containing the NR1 D481N and K483Q mutants; best-fit curves also demonstrate that Hill coefficients for both mutants were similar to that for the wild-type receptor.

The pharmacophore for the glycine binding site suggests that the binding of glycine involves interactions between a negative

TABLE 1

EC_{50} values for glutamate and glycine and maximum current amplitudes obtained with wild-type and mutant NR1 subunits

Concentration-response curves were obtained in the presence of a maximum concentration of coagonist. Data are mean ± standard error from the number of oocytes indicated (n); all mutants were tested in oocytes from at least two donor frogs.

Subunit combination	Glutamate EC_{50}	Glutamate n	Glycine EC_{50}	Glycine n	Maximum current size
	μM		mM		nA
Wild-type NR1a + NR2A	3.26 ± 0.77	12	2.33 ± 0.18	10	2400
NR1a subunit mutants + NR2A					
D481N	6.16 ± 0.67	4	16.27 ± 2.31	10	3000
K483Q	9.11 ± 1.13	4	293.3 ± 78.5	9	1600
D511N	3.46 ± 0.68	4	3.08 ± 0.7	4	2050
R523L	0	20	0	20	0
R448Q	4.66 ± 1.34	4	6.32 ± 0.62	4	1410
R489Q	1.10 ± 0.43	4	3.59 ± 0.65	4	1575
K531Q	1.21 ± 0.23	6	4.01 ± 0.41	4	2100
K544Q	5.13 ± 1.51	4	11.49 ± 1.91	4	2125
D481N/K483Q	5.82 ± 2.71	4	1548 ± 591	4	2300
K483A	2.16 ± 0.55	5	6.95 ± 1.13	5	3375
D481A	3.55 ± 1.27	4	375.3 ± 108.2	5	1800
D481K	4.11 ± 0.73	5	200.5 ± 66.1	6	2050

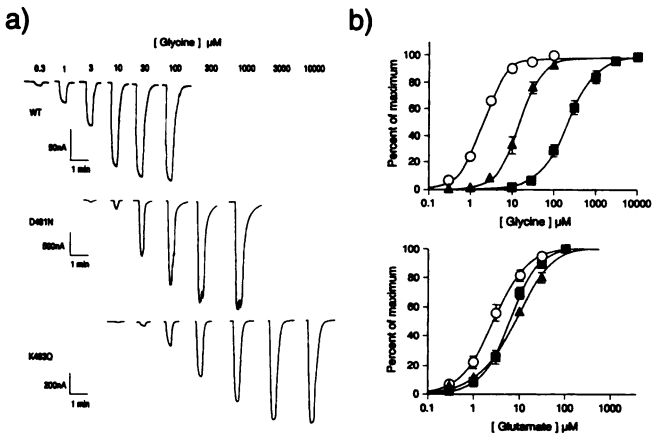


Fig. 2. a, Glycine-activated currents from oocytes expressing the human wild-type, D481N, or K483Q NR1 subunit together with human NR2A. *Numbers along the top*, glycine concentrations. *Calibration bars*, 1 min and currents indicated for each row. b, Normalized concentration-response curves for glycine (*top*) and glutamate (*bottom*) with wild-type (○), D481N (▲), and K483Q (■) receptors. Glycine concentration-response curves were obtained in the presence of 100 μM glutamate, and glutamate concentration-response curves were obtained in the presence of 100 μM, 1 mM, and 10 mM glycine. Data represent mean ± standard error for the number of oocytes stated in Table 1. *Curves* were fit using the Hill equation.

charge on the receptor and the amino group of glycine and between a positive charge on the receptor and the carboxyl moiety of glycine (3, 27). Therefore, one hypothesis is that the negatively charged aspartate (D481) might contribute the negative charge and the lysine at position 483 might contribute the positive charge that interacts with the carboxyl group of glycine. We tested this hypothesis by making additional mutations at these two positions to remove the side chain group. K483A had a glycine affinity similar to that of the wild-type receptor (Table 1), indicating that the basic side chain of this lysine probably does not interact with the carboxyl group of glycine. The mutation D481A, however, resulted in a 161-fold reduction of the glycine affinity (EC_{50} = 375 ± 108 μM), and changing the aspartate residue to a positively charged lysine (D481K) similarly reduced the glycine affinity approximately 100-fold (EC_{50} = 200 ± 66 μM). Again, these mutations had no affect on the affinity for glutamate (Table 1).

Partial agonists have lower affinity but similar efficacy with mutant NR1 D481N and K483Q subunits. To further explore the role of these amino acid residues, we determined the affinity and efficacy of several other glycine site agonists. Fig. 3 shows the concentration-response curves for the glycine site agonist D-serine and partial agonists D-cycloserine and ACBC. The affinity for these agonists was also significantly reduced with both mutants, although to a lesser extent than was that for glycine with the K483Q mutation (Table 2). Interestingly, the level of agonist efficacy, compared with glycine, was identical for wild-type NR1a/NR2A receptors and both mutants. D-Serine behaved as a full agonist (Fig. 3a), D-cycloserine reached a maximum of approximately 80% (Fig. 3b), and ACBC showed an unusually low efficacy for the NR1a/NR2A subtype and mutant NR1/NR2A, at 5% of maximum (Fig. 3c), compared with approximately 20% for native NMDA receptors expressed by rat cortical neurons in culture (28). The fact that the degree of efficacy for receptors containing mutated NR1 subunits was unchanged from that for the wild-type

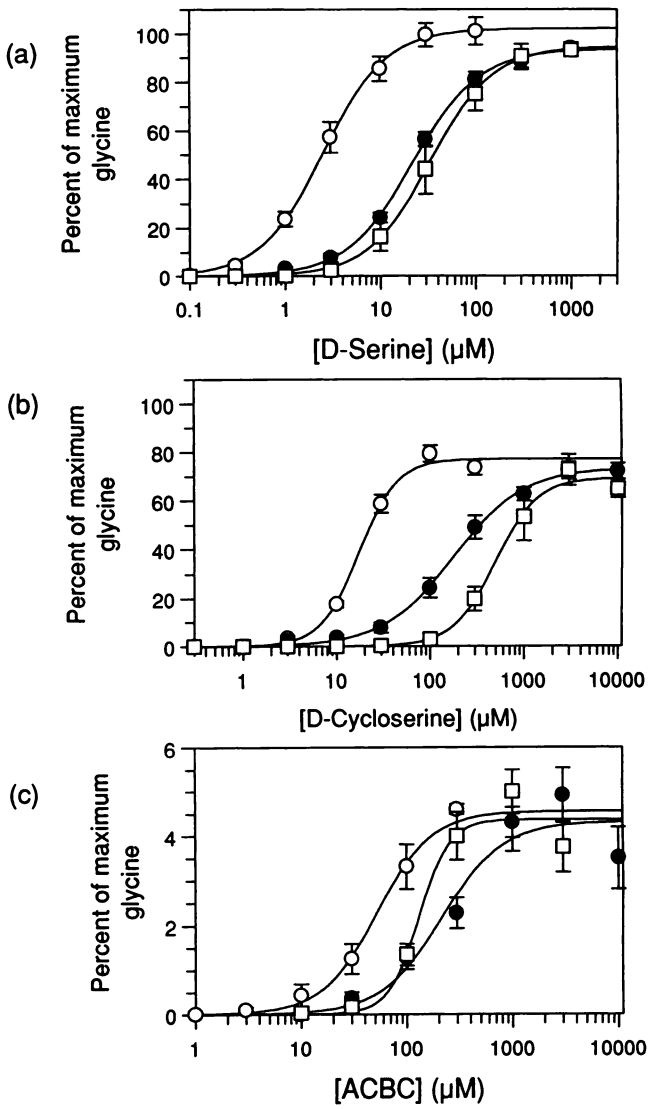


Fig. 3. Glycine site agonist concentration-response curves for D-serine (a), D-cycloserine (b), and ACBC (c) with wild-type (○), D481N (●), and K483Q (□) NR1 subunits combined with NR2A. Concentration-response curves were obtained in the presence of 100 μM glutamate, and agonist-induced currents were normalized to a maximum response to glycine for the same oocyte, to calculate efficacy. Using this method, D-serine was a full agonist (100% efficacy) and D-cycloserine and ACBC were partial agonists with efficacies of approximately 75% and 4.5%, respectively.

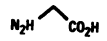
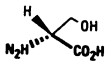
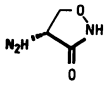
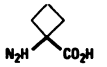
receptor also suggested that the mutations influenced the glycine binding site rather than subsequent gating events.

Antagonists have reduced affinity for receptors containing a mutant NR1 subunit. If D481 and K483 contribute to the glycine binding site, then it would be predicted that mutation of these residues would also lead to a reduction in antagonist affinity. To test this hypothesis we determined the affinities of two structurally diverse compounds, L-689,560 [(±)-4-*trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonyl-amino-1,2,3,4-tetrahydroquinoline] and the low efficacy partial agonist L-687,414 [(3*R*)-(+)-*cis*-4-methyl-HA-966], which have previously been shown to inhibit NMDA activity via the glycine binding site (29–31). Fig. 4 shows inhibition curves for these two compounds with wild-type receptors and the mutants D481N, K483Q, and D481A. Table 3 shows the K_i values of these compounds for inhibition of glutamate/glycine-gated cur-

TABLE 2

EC_{50} values of glycine site agonists and partial agonists for wild-type NR1 plus NR2A and the NR1 subunit mutants D481N and K483Q plus NR2A

Structures are indicated, with the glycine part of the molecule highlighted. Data are mean ± standard error of the number of oocytes indicated (*n*). Agonist EC_{50} values were calculated as described previously.

Glycine site agonist	Structure	Wild-type		D481N		K483Q	
		EC_{50}	*n*	EC_{50}	*n*	EC_{50}	*n*
		μM		μM		μM	
Glycine		2.33 ± 0.18	10	16.27 ± 2.3	10	293 ± 78.5	9
D-Serine		2.55 ± 0.12	5	22.2 ± 0.91	7	33.8 ± 1.10	6
D-Cycloserine		17.4 ± 1.3	6	176.9 ± 11.5	6	496 ± 47.2	4
ACBC		52.0 ± 6.4	4	214 ± 83	5	131 ± 30	7

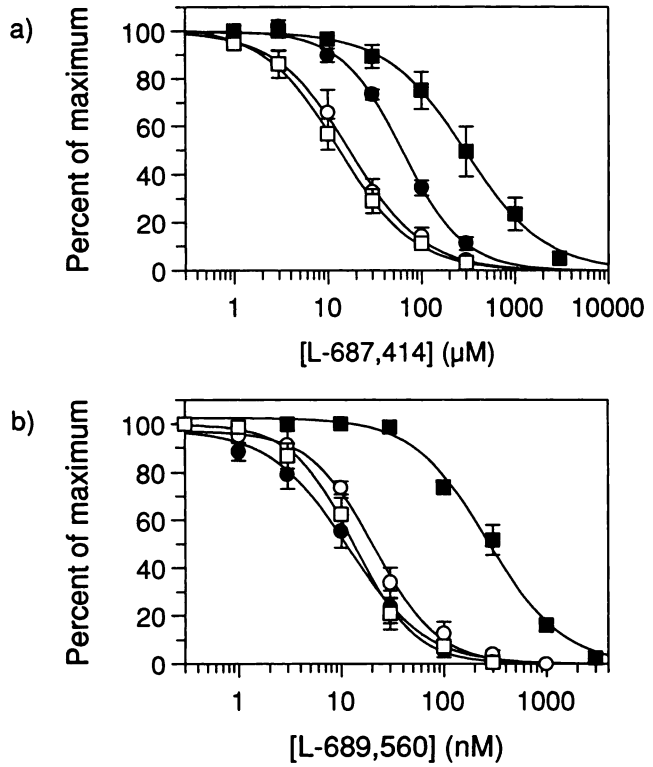


Fig. 4. Concentration-effect curves for glycine site antagonists with wild-type and mutant NR1 receptors. Inhibition curves for L-687,414 (a) and L-689,560 (b) were obtained with wild-type (○), D481N (●), K483Q (□), and D481A (■) NR1 subunits coexpressed with the human NR2A subunit. The data represent the mean of four to six oocytes, and the *curve* is fitted to the mean data. The IC_{50} values from individual oocytes were used to calculate the K_i values in Table 3. Where standard errors are not visible they are smaller than the plotted symbol. The concentration of glutamate present was 100 μM for all experiments. The concentrations of glycine used were as follows: wild-type, 10 μM; D481N, 100 μM; K483Q, 100 μM; D481A, 300 μM.

rents of wild-type and mutant receptors, calculated using the glycine affinity determined with the appropriate mutants. Affinity for the antagonist L-689,560 was only slightly reduced with the D481N and K483Q mutants (2–4-fold); however, the affinity for L-687,414 was more significantly reduced (13-fold) with the D481N mutant than the K483Q mutant (3-fold).

When L-687,414 and L-689,560 were tested with D481A, however, a much larger reduction in affinity than with D481N was observed (Fig. 4; Table 3). The affinity of L-689,560 was reduced 34-fold, with a K_i of 146.9 nM, and the affinity of L-687,414 was reduced 53-fold, with a K_i of 153.8 μM, suggesting that D481 does play a significant role in the binding of glycine site antagonists.

Discussion

Mutagenesis of amino acids in the human NR1 subunit has revealed D481 and K483 to be important residues involved in forming part of the active site for glycine and other glycine site agonists and antagonists. Experiments suggest that the aspartate (D481) interacts with the amino group of glycine or equivalent groups in other glycine site agonists and antagonists.

Similarly to a recent study that used mutagenesis to investigate agonist activation of the $GABA_A$ receptor (17), none of the NR1 mutants displayed reduced current amplitudes or Hill coefficients. Most importantly, the affinity for glutamate was relatively unchanged. These observations indicate that these amino acid mutations specifically affected the binding of glycine, rather than any downstream transduction process involved in activation of the channel or assembly of the receptor. Also, other agonists, including partial agonists, had efficacies with all of the tested mutants similar to those with wild-type receptors, supporting the hypothesis that these changes reduced affinity without affecting the degree of channel activation, as measured by agonist efficacy. One limitation of using *Xenopus* oocytes is the inability to observe rapid kinetic changes, such as those involved in receptor desensitization; hence, it is a possibility that observed changes in agonist affinity may be compromised by any effects of mutation on events such as

TABLE 3

Affinity of the glycine site antagonists L-687,414 and L-689,560 for wild-type NR1 plus NR2A and the NR1 subunit mutants D481N, D481A, and K483Q plus NR2A

Antagonist K_i values (mean ± standard error) were calculated from the glycine inhibition curves (IC_{50}) shown in Fig. 4, using the glycine EC_{50} values determined for the relevant mutants (Table 1). Log K_i values derived for individual oocytes were averaged for statistical analysis and are shown with the standard error as a range of values. The numbers of oocytes tested are indicated (*n*). Mutant K_i values were all significantly different from wild-type values ($p < 0.01$; K483Q with L-689,560, $p < 0.02$).

Subunit combination	L-687,414			L-689,560		
	K_i	*n*	Reduction in affinity	K_i	*n*	Reduction in affinity
	μM		fold	nM		fold
Wild-type NR1a + NR2A	2.9 (2.0–4.1)	4		4.2 (3.3–5.1)	4	
NR1a subunit mutants + NR2A						
K483Q	9.2 (7.5–11.3)	6	3	9.7 (7.7–12.2)	5	2
D481N	38.7 (36.2–41.4)	5	13	17.1 (12.5–23.2)	5	4
D481A	153.8 (105–223)	4	53	146.9 (124–174)	4	34

desensitization, which cannot be easily measured in *Xenopus* oocytes.

All glycine site agonists had significantly lower affinity for both D481N and K483Q (Fig. 3; Table 2) and, although the affinities were decreased approximately 10-fold for the D481N mutant, the decreases for the K483Q mutant were not as great as those for glycine itself. The affinity of L-687,414 was reduced 13-fold by the D481N mutation and even further by the D481A mutation (Table 3); this compound shares the amine in common with the agonists, rather than an amide as found in L-689,560, again suggesting some interaction at the amino group. These data show that, whereas the D481N mutation caused a modest but consistent reduction in agonist and antagonist affinity (2–13-fold), further removal of the side chain (D481A) or mutation of this residue to one possessing a positive charge (D481K) dramatically reduced both glycine affinity (161-fold for D481A and 86-fold for D481K) and glycine site antagonist affinity. This would be consistent with D481 contributing the negative charge to the glycine interaction. Mutation of the aspartate to asparagine (D481N) would still provide a carbonyl group in the amide moiety, which could form a hydrogen bond with the amino group of glycine, resulting in a weaker interaction with glycine (7-fold decrease). Removal of this side chain (D481A) would result in the loss of this interaction, reflected in the 161-fold decrease in glycine affinity and a 40–50-fold lower affinity for antagonists. It is likely that the smaller changes observed for antagonist affinity reflect the interactions of their larger structures with other parts of the receptor, particularly in the case of L-689,560, resulting in these molecules being less dependent on interactions with the amino acid residues that bind glycine itself. This is consistent with the pharmacophore model for the glycine agonist/antagonist site, adapted from the work of Kemp and Leeson (3) (Fig. 5).

If the K483 residue were contributing directly to the binding of glycine by providing the positive charge that interacts with the carboxyl group of glycine, we might expect the affinity for antagonists to be shifted to a greater extent with K483Q, because the carboxyl group of L-689,560 is critical for antagonist binding (27). The K483Q mutation clearly had a profound influence on glycine agonist affinity, but the relatively minor effect of mutation at this residue on antagonist affinity and the minimal effect on glycine affinity when the side chain was completely removed (K483A) suggest that this residue may not interact directly with glycine. However, its close proximity to D481 and F484 [F466 in the work of Kuryatov *et al.* (14)] and

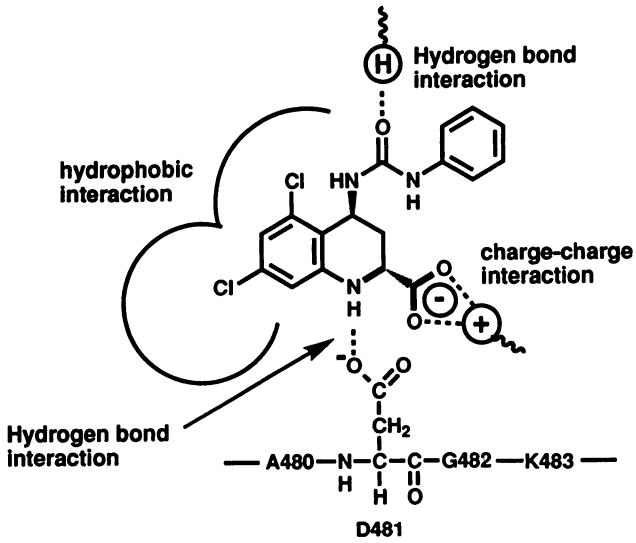


Fig. 5. Proposed pharmacophore model of the binding site for glycine/glycine site antagonists. The glycine site antagonist L-689,560 is illustrated, with the glycine substructure indicated in *bold*. The amide group of L-689,560 is shown with a putative hydrogen bond to the carboxyl group of the aspartate at position 481 of the NR1 subunit. This model was adapted from the work of Kemp and Leeson (3).

the large change in glycine affinity observed with the K483Q mutant suggest that this amino acid does influence the glycine recognition site but probably does not interact directly with glycine (Fig. 5). A previous study using similar mutagenesis techniques identified amino acids in this region as being involved in the putative glycine binding site (14). Those authors showed that mutation of aromatic residues in the region Q387–Y392 reduced glycine affinity and mutation of F466 to a nonaromatic residue produced similar large decreases in glycine affinity. These mutants were also much less sensitive to the glycine antagonist 7-chlorokynurenic acid. Based on these findings, Kuryatov *et al.* (14) proposed a secondary structural model similar to the bacterial amino acid-binding protein, forming an agonist binding pocket. Our findings are consistent with this model, inasmuch as residues D481 and K483 identified in this study (using the amino acid numbering of Refs. 9 and 19) correspond to D463 and K465 (using the numbering of Ref. 8), adjacent to F466 in this model. Kuryatov *et al.* (14) also found that mutations of D463 and K465 caused a reduction in glycine affinity; hence, this region is highly likely to contain the glycine binding pocket. Kuryatov *et al.* (14) expressed mutant NR1

subunits with the NR2B subunit, compared with the NR2A subunit used here. The finding that equivalent residues in the NR1 subunit reduce glycine affinity, whether the NR1 subunit is expressed with the NR2A or NR2B subunit, again provides additional evidence that the NR1 subunit contains the primary binding site for glycine. The question of which amino acids actually interact directly with the glycine molecule still remains. It is possible that both the aromatic residues identified by Kuryatov *et al.* and D481 identified in this study could contribute to the binding of glycine by interacting with the basic nitrogen. Indeed, such an arrangement has been reported for the interaction of phosphocholine with the antiphosphocholine antibody McPC603; crystallographic studies suggest that the basic triethylammonium group of phosphocholine interacts with three aromatic residues (two tyrosines and a tryptophan) and two negatively charged residues (a glutamate and an aspartate) (32).

Coexpression of the NR1 subunit with the NR2B or NR2C subunit produces receptors with approximately 10-fold higher glycine affinity than found with NR2A (33); hence, the NR2 variant can clearly influence the binding of glycine. However, the absence of agonist responses to homomeric expressed NR2 subunits and the formation of a high affinity glycine antagonist site in cells expressing NR1 alone (12, 13) suggest that glycine ligands interact directly with the NR1 subunit, and the NR2 subunits may act sterically in combination with NR1 to modulate glycine affinity.

The agonist binding sites of other ligand-gated ion channels, including the nicotinic receptor (15), the strychnine-sensitive glycine receptor (16), the $GABA_A$ receptor (17), and non-NMDA glutamate receptors (18), have also been explored using site-directed mutagenesis. Similarly to the data reported here for the NMDA receptor, in each case it has been found that the residues amino-terminal to the first putative transmembrane domain contribute to the agonist binding site. The most detailed studies have been performed with the *Torpedo* nicotinic acetylcholine receptor, where it is now clear from both site-directed mutagenesis and affinity labeling studies (15) that the residues that contribute to the agonist binding site are quite distantly located in the primary sequence of the α subunit, even though in the tertiary structure they are relatively closely associated. Thus, the residues that constitute the glycine binding site of the NMDA receptor are not necessarily located close to each other in the primary sequence. Indeed, other residues in the amino terminus and between proposed transmembrane regions 3 and 4 of the NR1 subunit also affect glycine affinity (14).

As well as having a glycine binding site, the NMDA receptor possesses a second coagonist site for glutamate, and both molecules are required to bind to allow ion channel activation. NR1 subunits expressed in *Xenopus* oocytes form channels that are sensitive to glutamate (in the presence of glycine) (4), suggesting that this site is also located, at least in part, on the NR1 subunit. Using the rationale described above, the glutamate binding site is also likely to be located in the amino-terminal region of the NR1 subunit. It is odd, however, that with all of the amino acids examined in this study and that of Kuryatov *et al.* (14), although several mutations affected glycine affinity, none of the mutations had any significant effect on glutamate affinity. Studies with the non-NMDA receptor GluR1 identified several amino acids, of which D443 and K445 are analogous to the residues D481 and K483 mutated in this study, that decreased the EC_{50} for glutamate, AMPA, kainate, and quisqualate (18); however, those workers were unable to determine whether the amino acid residues they identified were directly involved in agonist binding. Given the similarity of these subunits to the NR1 subunit, it may be that the glutamate binding site has been conserved between these homologous proteins and modified to bind glycine. It appears that amino acids in homologous regions of the amino-terminal regions of the glycine, nicotinic, and $GABA_A$ receptors (15–17) form at least part of the agonist binding sites for their respective agonists. The identification and characterization of agonist binding domains should contribute to the understanding of the structure and function of the superfamily of ligand-gated ion channels as a whole.

Acknowledgments

We would like to thank Dr. Paul Leeson for helpful discussions throughout the course of this work and P. Wingrove for his help with the mutagenesis.

References

1. Kleckner, N. W., and R. Dingledine. Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. *Science (Washington D. C.)* **241**:835–837 (1988).
2. Johnson, J. W., and P. Ascher. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature (Lond.)* **325**:529–531 (1987).
3. Kemp, J. A., and P. D. Leeson. The glycine site of the NMDA receptor: five years on. *Trends Pharmacol. Sci.* **14**:20–25 (1993).
4. Moriyoshi, K., M. Masu, T. Ishii, R. Shigemoto, N. Mizuno, and S. Nakanishi. Molecular cloning and characterization of the rat NMDA receptor. *Nature (Lond.)* **354**:31–37 (1991).
5. Monyer, H., R. Sprengel, R. Schoepfer, A. Herb, M. Higuchi, H. Lomeli, N. Burnashev, B. Sakmann, and P. H. Seeburg. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (Washington D. C.)* **256**:1217–1221 (1992).
6. Kutsuwada, T., N. Kashiwabuchi, H. Mori, K. Sakimura, K. Araki, H. Meguro, H. Masaki, T. Kumanishi, M. Arakawa and M. Mishina. Molecular diversity of the NMDA receptor channel. *Nature (Lond.)* **358**:36–41 (1992).
7. Ikeda, K., M. Nagasawa, H. Mori, K. Sakimura, K. Araki, M. Watanabe, Y. Inoue, and M. Mishina. Cloning and expression of the $\epsilon 4$ subunit of the NMDA receptor channel. *FEBS Lett.* **313**:34–38 (1992).
8. Meguro, H., H. Mori, K. Araki, E. Kushiya, T. Kutsuwada, M. Yamazaki, T. Kumanishi, M. Arakawa, K. Sakimura, and M. Mishina. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond.)* **357**:70–74 (1992).
9. Ishii, T., K. Moriyoshi, H. Sugihara, K. Sakurada, H. Kadotani, M. Yokoi, C. Akazawa, K. Shigemoto, N. Miszuno, and S. Nakanishi. Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J. Biol. Chem.* **268**:2836–2843 (1993).
10. Yamazaki, M., H. Mori, K. Araki, K. Mori, and M. Mishina. Cloning, expression and modulation of a mouse NMDA receptor subunit. *FEBS Lett.* **300**:39–45 (1992).
11. Sugihara, H., K. Moriyoshi, T. Ishii, M. Masu, S. Nakanishi. Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem. Biophys. Res. Commun.* **185**:826–832 (1992).
12. Lynch, D. R., N. J. Anegawa, T. Verdoorn, and D. B. Pritchett. *N*-Methyl-D-aspartate receptors: different subunit requirements for binding of glutamate antagonists, glycine antagonists, and channel blocking agents. *Mol. Pharmacol.* **45**:540–545 (1994).
13. Grimwood, S., B. Le Bourdellès, and P. J. Whiting. Human homomeric NMDAR1 receptors transiently expressed in mammalian cells form a high affinity glycine antagonist binding site. *J. Neurochem.*, in press.
14. Kuryatov, A., B. Laube, H. Betz, and J. Kuhse. Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins. *Neuron* **12**:1291–1300 (1994).
15. Changeux, J.-P., J. L. Galzi, A. Devillers-Thiery, and D. Bertrand. The functional architecture of the acetylcholine nicotininc receptor explored by affinity labelling and site directed mutagenesis. *Q. Rev. Biophys.* **25**:395–432 (1992).
16. Vandenberg, R. J., C. A. Handford, and P. R. Schofield. Distinct agonist and antagonist binding sites on the glycine receptor. *Neuron* **9**:491–496 (1992).
17. Amin, J., and D. Weiss. $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not pentobarbital. *Nature (Lond.)* **366**:565–569 (1993).
18. Uchino, S., K. Sakimura, K. Nagahari, and M. Mishina. Mutations in a putative agonist binding region of the AMPA-selective glutamate receptor channel. *FEBS Lett.* **308**:253–257 (1992).
19. Le Bourdellès, B., K. A. Wafford, J. A. Kemp, G. Marshall, C. Bain, A. S.

Wilcox, J. M. Sikela, and P. J. Whiting. Cloning, functional coexpression, and pharmacological characterization of human cDNAs encoding NMDA receptor NR1 and NR2A subunits. *J. Neurochem.* **62**:2091–2098 (1994).
20. Wingrove, P. B., K. A. Wafford, C. J. Bain, and P. J. Whiting. The modulatory action of loreclezole at the γ-aminobutyric acid type A receptor is determined by a single amino acid in the β2 and β3 subunit. *Proc. Natl. Acad. Sci. USA* **91**:4569–4573 (1994).
21. Wafford, K. A., P. J. Whiting, and J. A. Kemp. Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant γ-aminobutyric $acid_A$ receptor subtypes. *Mol. Pharmacol.* **43**:240–244 (1993).
22. Planells-Cases, R., W. Sun, A. V. Ferrer-Montiel, and M. Montal. Molecular cloning, functional expression and pharmacological characterization of an *N*-methyl-D-aspartate receptor subunit from human brain. *Proc. Natl. Acad. Sci. USA* **90**:5057–5061 (1993).
23. Karp, S. J., M. Masu, T. Eki, K. Ozawa, and S. Nakanishi. Molecular cloning and chromosomal localization of the key subunit of the human *N*-methyl-D-aspartate receptor. *J. Biol. Chem.* **268**:3728–3733 (1993).
24. Sun, W., A. V. Ferrer-Montiel, A. F. Schinder, J. P. McPherson, G. Evans, and M. Montal. Molecular cloning, chromosomal mapping, and functional expression of human brain glutamate receptors. *Proc. Natl. Acad. Sci. USA* **89**:1443–1447 (1992).
25. Kamboj, R. K., D. D. Schoepp, S. Nutt, L. Shekter, B. Korczak, R. A. True, T. M. Zimmerman, and M. A. Wosnick. Molecular structure and pharmacological characterization of humEAA2, a novel human kainate receptor subunit. *J. Neurochem.* **62**:1–9 (1993).
26. Laube, B., A. Kuryatov, J. Kuhse, and H. Betz. Glycine-glutamate interactions at the NMDA receptor: role of cysteine residues. *FEBS Lett.* **335**:331–334 (1993).
27. Leeson, P. D., R. W. Carling, K. W. Moore, A. M. Moseley, J. D. Smith, G. Stevenson, T. Chan, R. Baker, A. C. Foster, S. Grimwood, J. A. Kemp, G. R. Marshall, and K. Hoogsteen. 4-Amido-2-carboxytetrahydroquinolines: structure-activity relationships for antagonism at the glycine site of the NMDA receptor. *J. Med. Chem.* **35**:1954–1968 (1992).
28. Kemp, J. A., T. Priestley, and G. R. Marshall. Glycine site ligand affinity and efficacy: consequences for *N*-methyl-D-aspartate receptor modulation, in *Excitatory Amino Acids*. (B. S. Meldrum, F. Moroni, R. Simon, and J. H. Woods, eds.). Raven Press, New York, 213–222 (1991).
29. Kemp, J. A., T. Priestley, G. R. Marshall, P. D. Leeson, and B. J. Williams. Functional assessment of the actions of 4-methyl derivatives of HA-966 at the glycine site of the *N*-methyl-D-aspartate receptor. *Br. J. Pharmacol.* **102**:65P (1991).
30. Foster, A. C., J. A. Kemp, P. D. Leeson, S. Grimwood, A. R. Donald, G. R. Marshall, T. Priestley, J. D. Smith, and R. W. Carling. Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the *N*-methyl-D-aspartate receptor from rat brain. *Mol. Pharmacol.* **41**:914–922 (1992).
31. Foster, A. C., J. A. Kemp, M. D. Tricklebank, P. D. Leeson, R. W. Carling, B. J. Williams, S. Grimwood, A. R. Donald, T. Priestley, G. R. Marshall, K. Saywell, and R. Gill. Selective antagonists for the glycine site of the *N*-methyl-D-aspartate receptor. *Mol. Neuropharmacol.* **2**:97–102 (1992).
32. Dougherty, D. A., and D. A. Stauffer. Acetylcholine binding by a synthetic receptor: implications for biological recognition. *Science (Washington D. C.)* **250**:1158–1160 (1990).
33. Wafford, K. A., C. J. Bain, B. Le Bourdellès, P. J. Whiting, and J. A. Kemp. Preferential co-assembly of recombinant NMDA receptors composed of three different subunits. *NeuroReport* **4**:1347–1349 (1993).

Send reprint requests to: Keith A. Wafford, Neuroscience Research Centre, Merck, Sharp & Dohme Research Laboratories, Terlings Park, Eastwick Rd., Harlow, Essex CM20 2QR, UK.