Articles

Structural Basis for Understanding Structure−Activity Relationships for the Glutamate Binding Site of the NMDA Receptor

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Received November 2, 2001

We present new homology-based models of the glutamate binding site (in closed and open forms) of the NMDA receptor NR2B subunit derived from X-ray structures of the water soluble AMPA sensitive glutamate receptor. The models were used for revealing binding modes of agonists and competitive antagonists, as well as for rationalizing known experimental facts concerning structure−activity relationships: (i) the switching between the agonist and the antagonist modes of action upon lengthening the chain between the distal acidic group and the amino acid moiety, (ii) the preference for the methyl group attached to the α-amino group of ligands, (iii) the preference for the D-configuration of agonists and antagonists, and (iv) the existence of "superacidic" agonists.

Introduction

The NMDA receptor belongs to a family of ionotropic glutamate receptors and performs important functions in the central nervous system. It is involved in neuronal signaling processes, memory consolidation, and synaptic plasticity.[1,2] The neurotoxicity induced by NMDA hyperactivation leads to a number of pathological conditions, ranging from acute neurodegenerative disorders, such as stroke and trauma, to chronic neurodegenerative diseases, such as Huntington's disease, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis.[3−6]

At present, four sites at the NMDA receptor are considered as major targets for endogenous and exogenous ligands: (i) the glutamate site for agonists and competitive antagonists, (ii) the glycine site for coagonists and the corresponding antagonists, (iii) two or even three polyamine sites (voltage-dependent and voltage-independent, respectively) for NMDA receptor modulators, and (iv) the phencyclidine site for channel blockers (noncompetitive antagonists) (see refs 3−6). All of them display unique properties and pharmacology. This paper deals with the glutamate binding site as historically the first studied one and playing the key role in the work of the NMDA receptor. (For our recent studies on modeling the NMDA receptor ion channel and the phencyclidine binding site, see ref 7.)

A number of different agonists and antagonists have been synthesized for the glutamate binding site,[8−13] and some of them are shown in Figures 1 and 2. The structure−activity relationships for them include the following major regularities: the dependence of agonist (antagonist) activity on the length of the molecular chain, the preference for substitution on the nitrogen atom of the amino group, and the preference of the D-configuration for both agonists and antagonists.[5,14,15] It should, however, be mentioned that none of these compounds is currently used in medical practice, because of their adverse effects.[3,16,17] The latter circumstance dictates the necessity for further research with

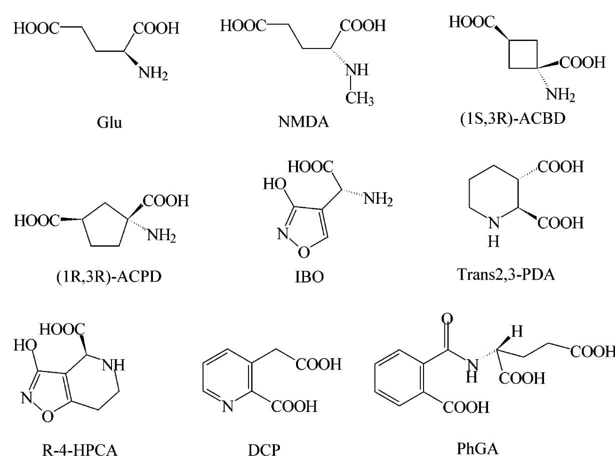

Figure 1. Agonists of the glutamate site of the NMDA receptor.

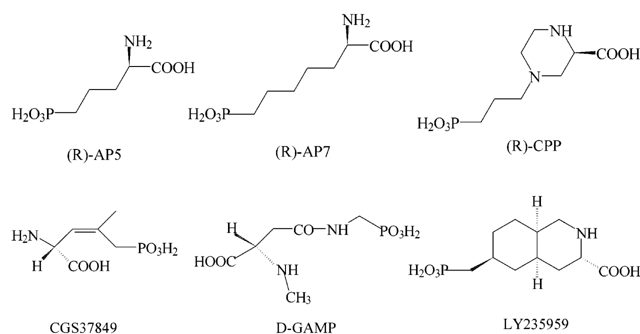

Figure 2. Antagonists of the glutamate site of the NMDA receptor.

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10.1021/jm011091t CCC: $22.00 © 2002 American Chemical Society
Published on Web 07/27/2002

the purpose of clarifying the mechanism of action and developing new ligands that would not cause undesirable side effects.

It has been shown that the NMDA receptor is composed of at least two NR1 and at least two NR2 subunits.[18−21] Each NR1 subunit is present in one of its splice variants, while each NR2 subunit belongs to one of its four subtypes, NR2A−NR2D, leading to a big heterogeneity of the wild-type NMDA receptors and variance of their electrophysiological properties and pharmacological profiles.[19] Four or five NR1 and NR2 subunits form an ion channel, which is usually blocked by magnesium in a voltage-dependent manner. Upon simultaneous activation of the receptor (under the action of an agonist on the glutamate site and a coagonist on the glycine site)[18] and the release of the magnesium blockage (caused by simultaneous partial depolarization of the membrane), an influx of calcium into the cell takes place. This effect is known to be the key element in the process of long-term potentiation underlying the memory formation.

All NR1 and NR2 subunits are characterized by the identical four domain structure: (i) the N-terminal extracellular modulatory domain (which contains a voltage-independent polyamine site); (ii) the agonist/coagonist binding domain, which holds the glycine binding site in an NR1 subunit and the glutamate binding site in an NR2 subunit; (iii) the transmembrane channel-forming region, which hosts several partially overlapping binding sites for magnesium, zinc, phencyclidine, polyamines (voltage-dependent site), etc.; and (iv) the C-terminal intracellular regulatory domain taking part in interactions with several intracellular proteins of postsynaptic density. In the 1990s, all genes encoding the NR1 and NR2 subunits were cloned and thoroughly studied, and their amino acid sequences were identified,[22,23] which offered the prospect of their comparative modeling. On the basis of the homology of amino acid sequences between bacterial periplasmic proteins and agonist binding domains of glutamate (both ionotropic and metabotropic) receptors, a common general structure and a common activation mechanism were postulated for their ligand binding domains.[24] It was assumed that such domains could consist of two lobes and exist in two forms, open and closed, while binding of agonists/coagonists causes closure of the domain (leading to receptor activation) via the Venus flytrap mechanism. Laube and colleagues[25] made the first attempt to build three-dimensional (3D) models of the NMDA receptor glutamate and glycine sites on the basis of sequence homology with the bacterial periplasmic protein (LAOBP). They have built an approximate model of these sites in the open form of the domain and suggested the mode of agonist and antagonist binding.

Meanwhile, in 2000, the X-ray crystallographic analyses of a water soluble AMPA sensitive glutamate receptor (AMPASGR), GluR2 ligand binding core, and its complexes with different ligands exhibiting agonist, partial agonist, and antagonist activity have been made.[26] Because the homology between AMPA and NMDA receptors is much higher (∼30%) than between LAOBP (∼18%) and the agonist/coagonist binding domains of the NMDA receptor and X-ray structures of open and closed forms of the AMPASGR are available, we suggested that 3D structures built by means of this homology would be more correct than the previous models and hence would allow us to shed light on mechanisms of receptor activation and to reach a better understanding of structure−activity relationships for NMDA receptor ligands.[5,14,15]

In this study, molecular modeling of the glutamate site belonging to the NR2B subunit of the NMDA receptor has been carried out (for our preliminary communications, see ref 27). The obtained models were used for studying different modes of ligand binding and explaining known structure−activity relationships for its agonists and competitive antagonists. The work with the glycine site is also in progress in our laboratory and will be a matter of a separate publication.

Materials and Methods

The multiple amino acid sequence alignment was built by means of the Clustal X program.[28] The BLOSSUM30 homology matrix was used for evaluating amino acid similarity. This sequence alignment was used as a basis for homology modeling using the Biopolymer module in the Sybyl6.6 molecular modeling package[29] on the SGI Octane workstation. The X-ray structure of the complex of the AMPASGR with glutamate (Brookhaven Protein Data Bank[30] accession code 1FTJ) was used as a template for modeling the closed form of the glutamate binding site of the NMDA receptor, while the X-ray structure of the complex of the same protein with an antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX, Brookhaven Protein Data Bank[30] accession code 1FTL) was used as a template to model the open form of the same binding site.

In the course of molecular modeling, a template protein was mutated to a goal protein in accordance with the following procedure. All aligned nonidentical amino acids were "mutated" to each other by changing their side chains while keeping main chain atoms unchanged and fixed at their original position in space. Internal torsion angles in side chains of new amino acids were chosen to be most appropriate for a given amino acid in a given secondary structure state (which remains unchanged while "mutating" residues). All insertions and deletions were handled by defining "loops" containing all residues being inserted and several atoms neighboring to positions of insertion or deletion, followed by searching the Brookhaven Protein Data Bank[30] for them. After that, search results were visually inspected, and the main chain atoms of those loops, which (i) the geometry of the main chain atoms neighboring to the deletion or insertion positions underwent smaller changes, (ii) the amino acid sequence possessed the strongest homology to the required sequence, and (iii) no unfavorable spatial hindrance between atoms belonging to the loop and to the remaining part of the protein could be expected, were inserted in the protein model followed by adding appropriate side chains in accordance with the aforementioned procedure. After that, all proline residues and side chains of all other residues were "fixed" by finding new values for several torsion angles so as to remove spatial overlap between atoms. This was followed by adding hydrogen atoms capable of forming hydrogen bonds. After that, the protein was subjected to energy minimization using the Tripos force field as implemented in the SYBYL package.[29] Then, the protein was solvated with a box of water molecules and reminimized with the AMBER 6.0[31] program SANDER using the Cornell et al. force field.[32] This was followed by a short 20 ps molecular dynamics simulation at 300 K using the same SANDER program. The complexes of the protein with D-AP5 and L-Glu were subjected to longer molecular dynamics simulations (100 ps).

The stereochemical quality of the protein models was checked using the PROCHECK program[33,34] with a pseudo-resolution of 1.9 Å. The sequence−structure compatibility of the protein models was assessed using the Verify3D[35] program.

Figure 3. Sequence alignment used for building models.

Ligands. All ligands were built interactively using Sybyl6.6. The carboxyl and amino groups of ligands were ionized, since in this form they usually occur under physiological conditions. Atomic charges in ligands were computed with the Gasteiger–Marsili method.[36]

Docking. A putative binding pocket was determined on the basis of known data on site-directed mutagenesis.[25] Each ligand was placed into the binding pocket and orientated taking into account the X-ray structures of the AMPASGR–ligand complexes. A manual docking procedure was applied, and the obtained receptor–ligand complexes were optimized using the Tripos force field. Different conformations and orientations of each ligand within the binding pocket were explored, and each time, the ligand–protein complex was reminimized.

Results and Discussion

Alignment of Amino Acid Sequences. The multiple amino acid sequence alignment for modeling the glutamate site of the NMDA receptor was built between the primary sequences of all representatives of the ionotropic glutamate receptors family, namely, NR1 (Swiss Protein Data Bank[37] accession code P35437), NR2A (Q08948), NR2B (Q13224), NR2C (Q14957), NR2D (Q15399), GLUR1 (P42261), GLUR2 (P42262), GLUR3 (P42263), GLUR4 (P48058), GLUR5 (P39086), GLUR6 (Q13002), KA1 (Q16099), and KA2 (Q16478) subunits and the sequence of the water soluble AMPASGR. A section of this alignment containing only the primary sequences of the NR2B subunit (chosen by us for modeling the glutamate site of the NMDA receptor) and AMPASGR (serving as a template for comparative modeling) and spanning the sequence region homologous to the whole molecule of AMPASGR is depicted in Figure 3. The whole multiple alignment of all sequences can be obtained in the Supporting Information. It is noteworthy mentioning that the use of the pairwise alignment between the latter two sequences instead of the multiple sequence alignment between all subunits of ionotropic glutamate receptors does not lead to construction of a correct molecular model, since it does not detect the transmembrane channel-forming region inside a primary sequence of the NR2B subunit.

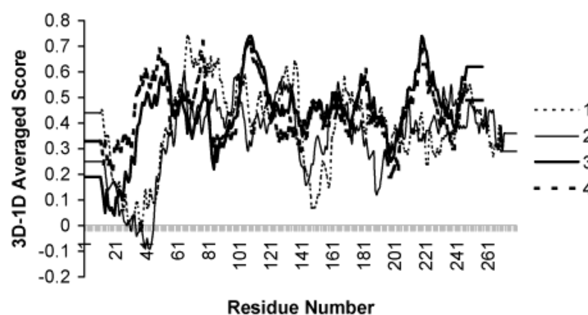


Figure 4. Verify 3D plots of the models of the NMDA receptor glutamate site closed (1) and open (2) forms in comparison with X-ray structures of AMPASGR complexes with an agonist (3) and antagonist (4).

Assessment of Models Quality. Analysis of the model in open and closed forms using PROCHECK[33,34] revealed good stereochemistry. Standard deviations of all main chain and side chain stereochemical parameters were within or better than the expected values;[33] ~73% of the residues in the closed form and ~70% in the open form adopted the most favored main chain torsion angles, and only ~1% (in the open form) and ~2% (in the closed form) of residues lied in unfavorable regions. No short intramolecular contacts were detected. Energy profiles analysis using Verify 3D[35] displayed that negative score regions (30–31 (432–433), 34–36 (436–438), 37–45 (439–446)) were found in the model, but they were localized at the loops far from the binding sites that could not influence on the validity of the structural sites affecting ligand selectivity (Figure 4).

Structure of Receptor Binding Site. On the basis of the sequence homology with AMPASGR, 3D models of the glutamate site in the open and closed forms were obtained. It is common knowledge that receptors exert their biological action by changing their conformations upon binding ligands–agonists. However, the issue of the exact number of such conformations is a matter of hot discussion.[38,39] Although the most popular point of view implies the existence of two main conformations,[40]

a ligand binding "active" and a ligand-free "nonactive" one, numerous works suggest the existence of multiple (or even a continuum of) conformational states involved in receptor activation.[38,39] An X-ray study of the AMPASGR complexes with different ligands suggests the existence of at least three principal conformational states[26] of the ligand binding region: (i) "closed" conformations adopted by complexes with agonists, (ii) "half-open" conformations stabilized by partial agonists, and (iii) "open" conformations in the ligand-free form and in complexes with antagonists. The main distinction between these forms is expressed by an angle between two lobes comprising the protein globule forming the ligand binding domain. However, comparison of X-ray structures of AMPASGR complexes with two AMPA receptor agonists, glutamate and AMPA, reveals a rather small difference between them (rms of fitting between the complexes is only 0.66 Å for all atoms), so one can suggest that it is sufficient to use only one conformation of the glutamate binding site for modeling its interactions with agonists. The difference between open conformations is even smaller (rms of fitting between the ligand-free "apo" and DNQX bound forms is 0.55 Å for all atoms), so we assume that considering only one conformation of this site is also sufficient for modeling its interactions with antagonists. So, in accordance with the principle of Ockham's Razor,[41] we conclude that the model with two conformational states, one for complexes with agonists and one for complexes with antagonists, may be adequate for pharmacology studies and therefore can be adopted for the purposes of this work.

In this study, the X-ray structure of the complex of AMPASGR with glutamate (access code 1FTJ in the Brookhaven Protein Data Bank[30]) was used for modeling the closed agonist binding form of the glutamate site of the NMDA receptor, while the X-ray structure of the complex of AMPASGR with AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (access code 1FTL in the Brookhaven Protein Data Bank[30]) was used for modeling the antagonist binding open form of the same site. Molecular modeling has shown that the glutamate binding domain of the NMDA receptor consists of two lobes composed of β-sheets covered with α-helices. In the model, these two lobes are connected with a hinge region (Figure 5a,b). In a real receptor, there is also a transmembrane channel-forming region coming from one of these lobes, so the switch between the open and the closed states of the glutamate binding domain causes the switch between the open and the closed states of the NMDA receptor ion channel through an indirect mechanism involving mutual rotation of different subunits (see Discussion in ref 26). Agonist-induced closure of the glutamate site leads to the activation of the receptor and opening of the channel, while the antagonist-induced opening of the same site leads to the closure of the channel.

Binding Modes of Agonists and Antagonists. In accordance with site-directed mutagenesis experiments,[25] amino acid residues E413, K485, S512, R519, S690, T691 in NR2B are important for receptor functioning, and this may take place if they are either involved in binding agonists or in gating mechanism, or they are important for proper protein folding.[42] From

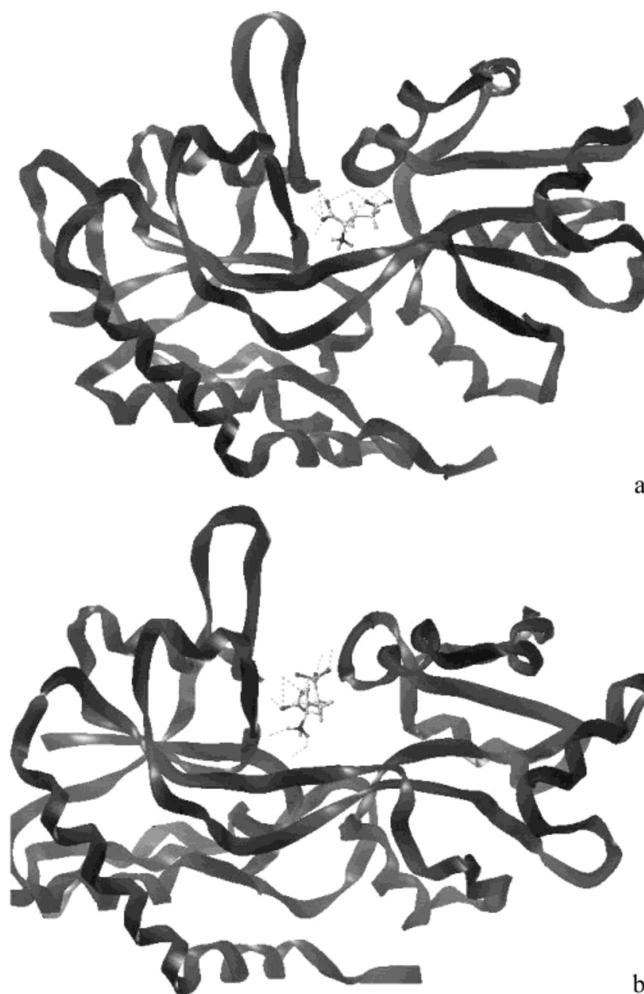


Figure 5. Binding of agonist glutamate (a) and antagonist R-AP5 (b) to the NMDA receptor glutamate binding site after molecular dynamics simulation.

the analysis of the binding mode of AMPASGR with glutamate, one can reveal residues forming hydrogen bonds and ion bridges with ligand (R94 in AMPASGR interacts with α-carboxyl group, E191 and P87 are bonded to amino group, and S140 and T141 bind to γ-carboxyl group). Considering the positions of the corresponding amino acid residues in the multiple alignment, it can be found that residue R519 in NR2B (corresponds to R94 in AMPASRG), S690 (S140), and T691 (T141) are conservative in NR2B and AMPASGR; however, S512 in NR2B corresponds to P87 in AMPASGR. Residue E191 in AMPASGR, which participates in hydrogen bond formation with the amino group of glutamate, corresponds to D732 in NR2B (no mutagenesis data have been reported for this case). All of these facts were taken into account when docking the agonists and antagonists shown in Figures 1 and 2 to this site. These docking experiments revealed that the optimal orientation could be achieved when the α-carboxyl group of agonists was allowed to form a salt bridge with R519 and hydrogen bonds with S690 and T514, while the protonated amino group is hydrogen-bonded to E413 and S512, in accordance with Laube's et al. model.[25] However, the distal acidic function of agonists, in contradiction to Laube's et al. model,[25] is predicted in our model to form hydrogen bonds with S690 and T691. One can suggest that the difference between

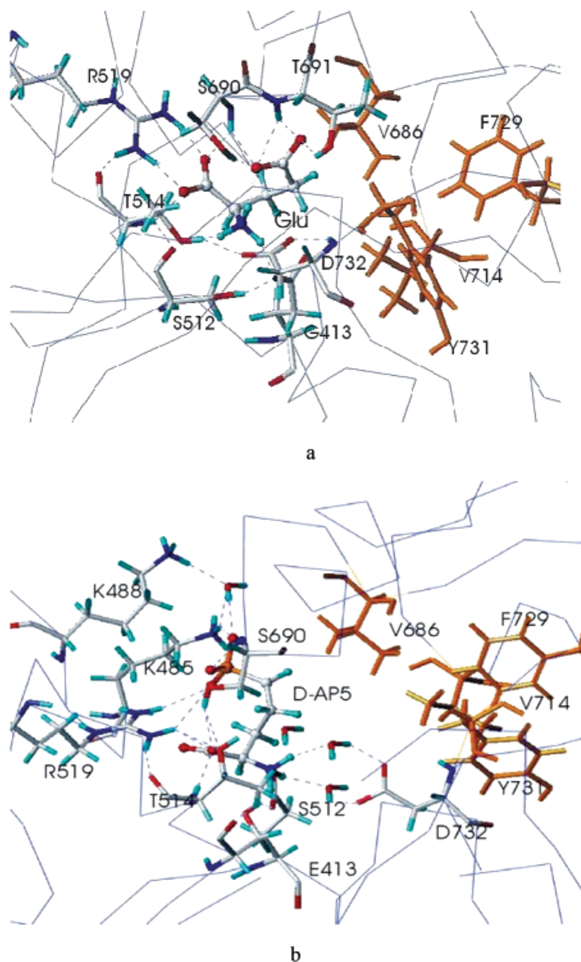


Figure 6. Binding mode of agonists (a) and antagonists (b) to the NMDA receptor glutamate site.

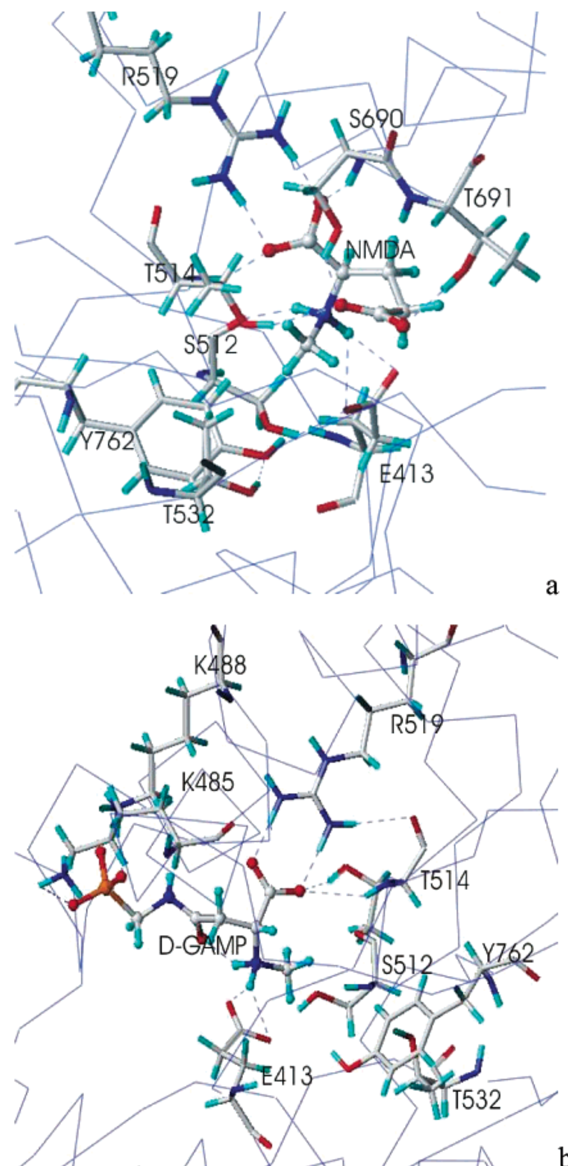
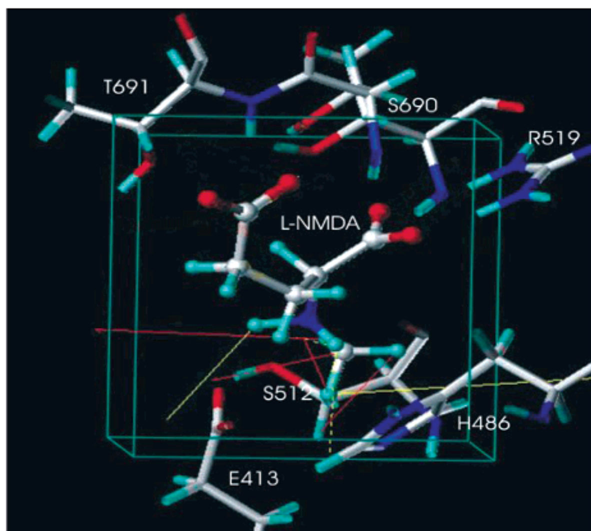


Figure 7. Binding of NMDA (a) and D-GAMP (b) to the glutamate site.

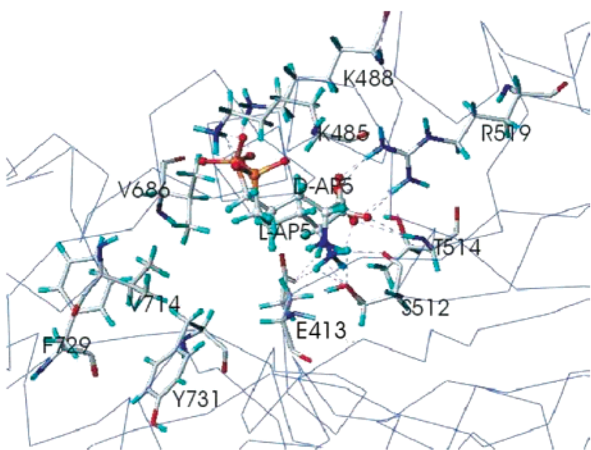
Laube's et al. and our models might partially be accounted for by the fact that Laube et al. built all of their models, including even complexes with agonists, for the open form of the glutamate binding site, although in reality agonists make stable complexes with the closed form. In the case of antagonists, we have found that the α-acidic moiety and amino group interact with the same residues as agonists, whereas a distal group (phosphonic in R-AP5) forms salt bridges with K485 and K488.

To improve results of docking and study the role of water in ligand binding, we have performed molecular dynamics studies of protein complexes with glutamate and AP5 (Figure 6a,b). The results of these studies have shown that in both cases the amino group interacts preferably with D732, while the docking procedure predicts binding of this group with E413 (see above). Both residues are located in the vicinity of this amino group but at opposite sides. It is possible that both residues contribute to binding this group; however, the 100 ps dynamics did not reveal a reverse switch from D732 to E413. The molecular dynamics simulations have also allowed refinement of the position of the distal group in AP5: it forms a hydrogen bond with the main chain NH moiety belonging to S690, and it is also bound to K485 directly and to K488 through a water molecule. Different interaction modes result in dissimilar arrangement of agonists and antagonists in the glutamate binding domain. Figure 5a,b illustrates the general arrangement of agonists and antagonists inside the glutamate site for the cases of glutamate and R-AP5. Glutamate, the native agonist, lies deeply in the binding pocket approximately in parallel with the hinge region, interacts with both lobes of the domain, and causes them to approach each other (see Figure 5a). An antagonist R-AP5 is, on the contrary, oriented perpendicularly with respect to the hinge region, and its distal group prevents the lobes from closure (see Figure 5b). Detailed analysis of the binding pocket made it possible to account for the structure−activity relationships discovered in various pharmacological and electrophysiological studies.[5,14,15]

Switch between the Agonist and Antagonist Activity of Ligands upon Lengthening the Chain between the Amino Acid Moiety and the Distal Anionic Group. The dependence of the agonist and antagonist activity of ligands acting on the glutamate site of the NMDA receptor upon the distance between two acidic groups was discussed in the literature.[5] In particular, a shorter distance is needed for a ligand to exhibit agonist activity, while antagonists are characterized by a longer distance between them (compare



a



b

Figure 8. Docking of NMLA (a) and L-AP5 (b) to the glutamate site.

formulas in Figures 1 and 2). This fact can easily be explained on the basis of molecular models. It is seen in Figure 6a that the distal carboxyl of the native agonist glutamate lies in the vicinity of a small pocket formed by four hydrophobic residues: V714, Y731, F729, and V686. One can suggest that the wall of this pocket limits the size of agonists. Antagonists do not fit to the size of the binding pocket, so they have to turn around and bind to K485 and K488 (see Figure 6b). This might explain the switch between the agonist and the antagonist activity of ligands upon lengthening the chain between the amino acid moiety and the distal anionic group. It can also be predicted from this study that it is impossible to find a lengthy or a bulky agonist for this binding site.

Preference for Substitution at the Nitrogen Atom in the Amino Group. Using our models, one can explain the following structure−activity relationship: the preference for substitution at the nitrogen atom in the amino group for both agonists and antagonists. The binding of NMDA and D-GAMP to the glutamate site is shown in Figure 7a,b. In both cases, there is a small hydrophobic pocket for the CH_3 group formed by Y762 and T514. This might explain this experimental fact.

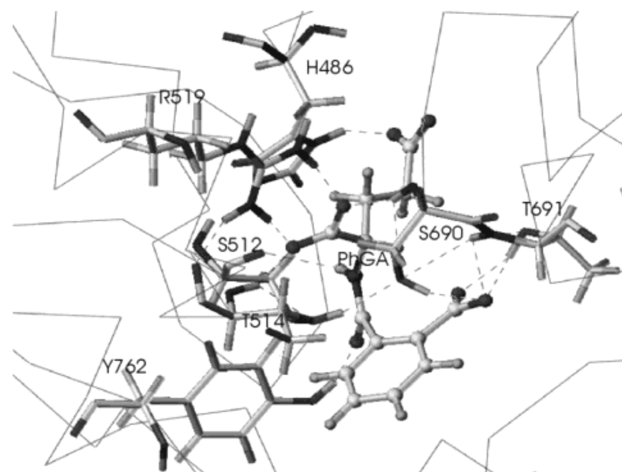


Figure 9. Binding of PhGA to the glutamate site.

Preference for the D-Configuration in Agonists and Antagonists. To explain the preference for the D-configuration of the chiral center in both agonists and antagonists, L-forms of agonist NMDA (i.e., NMLA and N-methyl-L-aspartate) and antagonist AP5 were docked to the models of the binding site (Figure 8a,b). We have found that the reason for preference for the D-configuration is different for agonists and antagonists. For the case of agonists, there is a steric repulsion between NMLA and residues H486 and S512 that hinders binding. Comparing binding of L-AP5 (Figure 8b) and D-AP5, it can be noted that the distal negatively charged phosphonic group in the D-configuration is closer to positively charged K485 and K488 than for the L-configuration. So, electrostatic ligand−protein interaction is more favorable for the D-configuration of antagonist AP5.

Superacidic Agonists. In this study, we have suggested the binding mode of selective superacidic agonist N-phthalamoyl-L-glutamic acid (PhGA).[5] This compound does not possess the pharmacophore (consisting of two acidic and one amino group) that is common for almost all ligands of the glutamate site of the NMDA receptor. Using models, we can see the important role of the carboxyl group attached to the benzene ring. This group plays the role of the distal acidic group of agonists and binds to S690 and T691 (see Figure 9). This explains the crucial role of this group for binding to the NMDA receptor known from the experiment.[9]

Receptor Subtype Specificity between Different NR2 Subunits. At the present time, not one of the known ligands of the glutamate site is selective with respect to different NR2 subunit subtypes. Examination of molecular models can explain this fact. The distance between the ligands and the nearest nonconservative among NR2A−D subtype amino acids (G713 in NR2B) exceeds 7.5 Å, and there is no free space between them (they are screened by conservative V714). So, it seems that it is hardly possible to design subtype selective ligands acting on the glutamate site.

Conclusion

Relatively high homology within the group of glutamate ionotropic receptors allowed us to use the results of the X-ray analysis of AMPASGR for molecular modeling of the NMDA receptor glutamate site. The models

obtained satisfactorily explain the structure–activity relationships for binding a series of agonists and competitive antagonists of the glutamate site. These models can reveal the key residues that interact with ligands. Further studies in this direction would validate these models, which could subsequently be used for de novo drug design. Coordinates of the model in open and closed forms will be submitted to the Brookhaven Protein Data Bank.

Acknowledgment. We thank Tripos GmbH (Münich, Germany) and the V. N. Orekhovich Institute of Biomedical Chemistry of the Russian Academy of Medical Sciences for scientific and technical assistance. We acknowledge the University of California, San Francisco for providing Amber 6.

Supporting Information Available: The whole multiple alignment of all sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM011091T