An Alanine Residue in the M3-M4 Linker Lines the Glycine Binding Pocket of the N-Methyl-D-aspartate Receptor*

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While attempting to map a central region in the M3-M4 linker of the N-methyl-D-aspartate receptor NR1 subunit, we found that mutation of a single position, Ala-714, greatly reduced the apparent affinity for glycine. Proximal N-glycosylation localized this region to the extracellular space. Glycine affinities of additional Ala-714 mutations correlated with side chain volume. Substitution of alanine 714 with cysteine did not alter glycine sensitivity, although this mutant was rapidly inhibited by dithionitrobenzoate. Glycine protected the A714C mutant from modification by dithionitrobenzoate, whereas the co-agonist l-glutamate was ineffective. These experiments place Ala-714 in the glycine binding pocket of the N-methyl-D-aspartate receptor, a determination not predicted by previous structural models based on bacterial periplasmic binding protein homology.

The N-methyl-D-aspartate (NMDA) receptor is distinguished among the ionotropic glutamate receptors by several properties. The receptor possesses a unique Ca2+-permeability and a voltage-dependent Mg2+ block (1). These two features define the NMDA receptor's role in several important physiologic phenomena, including long term potentiation, neuronal development, and excitotoxicity (2). However, the functional characteristic that may most differentiate the NMDA receptor from other glutamate receptors is a requirement for the co-agonist glycine (3, 4). Occupancy of both glutamate and glycine binding sites is necessary for receptor activation, and the two sites are allosterically coupled (5, 6). The distinct glycine binding site has been suggested to play a role in regulation of NMDA receptor desensitization (7). While several residues in the amino terminus of NR1 have been demonstrated to greatly influence glycine affinity, binding is also modulated by the interacting NR2 subunits (8). Indeed, the glycine binding site is a current experimental target for therapeutic intervention in the hours immediately following ischemic stroke (9).

Despite functional differences among the glutamate receptors, several recent studies have converged upon a single topology model for the ionotropic glutamate receptor family (10–14). Speculation of alternate glutamate receptor topology was suggested by several early investigations (15–19). Several subsequent systematic studies concluded that non-NMDA glutamate receptor subunits possessed a three-transmembrane domain architecture (11, 12, 20). By generating functional N-linked glycosylation mutants of the NMDA NR1 subunit, we tested the hypothesized existence of a conserved hairpin pore structure similar to that found in voltage-gated K+ channels (13), thus supporting the three-transmembrane domain model. An important consequence of the new three-transmembrane domain configuration is an extracellular location of the M3-M4 linker.

Inconsistent with the conclusion from these topology studies, however, were findings from several groups that residues in the M3-M4 linker of non-NMDA glutamate receptors could be phosphorylated (21–25). Most notably, two separate laboratories had determined that a serine residue (Ser-684) in the M3-M4 linker of GluR6 acted as a substrate for protein kinase A (PKA) (21, 22). The topology studies and the GluR6 phosphorylation data may be reconciled if two short transmembrane sections flanking the phosphorylation site are invoked (Fig. 1; see also Ref. 13). We have investigated this possibility utilizing the NR1 subunit through the introduction of both a novel PKA site and an N-linked glycosylation site in a region analogous to Ser-684 of GluR6. Subsequently, we have identified an alanine residue present in the glycine binding pocket of the NMDA receptor.

Experimental Procedures

Site-directed Mutagenesis—Mutagenesis was performed using the mega-primer method as described previously (13). Mutations were cloned into the pCR vector (Invitrogen) prior to insertion into a modified pN60 NR1 clone (26) containing an altered 5′-untranslated region (27) using the restriction enzymes Sacl (nucleotide 2058) and Sphl (nucleotide 2237) (New England Biolabs). Mutations were verified by DNA sequencing (U. S. Biochemical Corp.).

cRNA Synthesis and Oocyte Expression—Quantitative cRNA injections were performed as described (27). The construct e68 (mouse NR2A with the 5′-untranslated region truncated at nucleotide 68) was used with the coinjection experiments (28). Two separate transcription reactions of each cRNA were performed, and four batches of oocytes were injected (two of each transcription). In each case, five oocytes of each combination were tested 3 days after injection. Otherwise, cRNA was in vitro synthesized as described previously (13). All measurements were made from oocytes cojected with robustly expressing e constructs 2–5 days after injection (27).

Electrophysiology—Whole cell responses to agonist applications were obtained as described previously (13). Oocytes were held at −50 mV and perfused continuously with a Mg2+- and Ca2+-free solution containing (in mM) 100 NaCl, 5 KCl, 0.5 BaCl2, and 10 HEPES, pH 7.4, 24 °C. Responses to submaximal agonist concentrations were preceded and followed by measurements at maximal concentrations to correct for any drift caused by gradual run-up of oocyte responses. Peak current responses to agonist application and accompanying voltage measurements were determined using PCLAMP hardware and software (Axon Instruments, Burlingame, CA). Dose-response curves for l-glutamate and glycine were fitted with the Hill equation with the Hill coefficient fixed at unity. Half-maximal concentrations (EC50) were calculated by minimizing the sum of squared residuals calculated from the averaged responses at each concentration. Glycine-apparent affinities were

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1 The abbreviations used are: NMDA, N-methyl-D-aspartate; PKA, protein kinase A; DTNB, dithionitrobenzoic acid.
Asp-161, of LAOBP.

by the artificial G3mutation. This may be due to disruption of the antigenic epitope (monoclonal antibody 54.1, Pharmingen) produced a weaker signal of ployclonal antibody because the signal produced using the generated against the carboxyl terminus of NR1a (Chemicon) was employed as primary antibody. Western analysis was also performed as described previously (13), except that the polyclonal antibody was generated as described previously (13). Western analysis was also performed as described previously (13). Western analysis was also performed as described previously (13).

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RESULTS

Introduction of a Novel PKA Site—A canonical PKA consensus sequence was designed around an existing serine in the M3-M4 linker of NR1 at position 714 (NR1, P675A; G2, S773A (15)) and one located near the center of the linker (G3, this paper) are shown. The location of putative phosphorylation sites are also indicated. Beneath the topology diagram, an alignment of the NR1 sequence with several glutamate receptor subunits is shown (26, 52–55).

FIG. 1. Topology and homology of ionotropic glutamate receptors. Two functional N-linked glycosylation sites located at the extremes of the M3-M4 linker in the NR1 subunit (G1, P675A; G2, S773A (15)) and one located near the center of the linker (G3, this paper) are shown. The location of putative phosphorylation sites are also indicated. Beneath the topology diagram, an alignment of the NR1 sequence with several glutamate receptor subunits is shown (26, 52–55). Homologous residues appear in boldface type. Putative phosphorylation targets are shown with a black background: Ser-684 in GluR6 (21, 22), GluR2 (44) in this figure. The closed arrow indicates the ligand contact residue, Arg and Leu) deleted from the alignment of Sutcliffe et al. (44) in this figure. The closed arrow indicates the ligand contact residue, Aasp-161, of LAOBP.

measured in the presence of 100 mM L-glutamate and L-glutamate affinities in the presence of 100 mM glycine.

Western Analysis—Membranes from 30 Xenopus oocytes were prepared as described previously (13). Western analysis was also performed as described previously (13), except that a polyclonal antibody generated against the carboxyl terminus of NR1a (Chemicon) was employed as primary antibody because the signal produced using the monoclonal antibody generated against the M3-M4 linker of NR1 (monoclonal antibody 54.1, Pharmingen) produced a weaker signal of the G3 mutation. This may be due to disruption of the antigenic epitope by the artificial N-glycosylation site.

mutated residues were interfering with glycine binding.

To identify which of the four mutated residues comprising the artificial PKA site were responsible for the shift in affinity, we generated the individual point mutants of each residue. Fig. 4A demonstrates that mutation of the alanine at position 714 to leucine (714L) produced the dominant shift in glycine affinity. Furthermore, another reconstructed PKA mutation including an alanine reversion at position 714 (NR1 710:RRASL) restored most of the glycine sensitivity (EC50 = 17 μM, see Fig. 4B). Therefore, position 714 is the most influential residue within the artificial PKA site affecting apparent glycine affinity. This effect may be interpreted to indicate that the presence of a leucine at position 714 interferes with ligand binding and that Ala-714 therefore is part of the glycine binding site. However, there are two problems that complicate the localization of a mutated residue to an agonist binding site: transduction and

FIG. 2. Reduced glycine sensitivity in NR1-PKA. Responses of (A) wild type (NR1 + NR2A), and (B) mutant (NR1-PKA + NR2A) receptors expressed in Xenopus oocytes are shown. Responses to 100 μM L-glutamate + 1 μM glycine are shown flanked by responses to 100 μM L-glutamate + maximal glycine concentrations (100 μM and 10 mM, respectively). Open bars above the trace indicate perfusion of the agonist-free solution, and filled bars indicate agonist application. C, dose-response curves of the wild type (filled circles) and mutant (open circles) receptors. Mean responses are shown with standard errors of the mean (S.E.) when larger than the symbol. Calculated EC50 values are reported in Table I.

Ser-713 is not required for reduction in glycine sensitivity in NR1-PKA.

Glycine responses of the mutant receptor NR1 710:RRAAL were obtained as in Fig. 2. Filled symbols represent means with S.E. Curves of wild type NR1 (dashed line) and NR1-PKA mutant (thin line) receptors are redrawn from Fig. 2 for comparison.

FIG. 3. Ser-713 is not required for reduction in glycine sensitivity in NR1-PKA. Glycine responses of the mutant receptor NR1 710:RRAAL were obtained as in Fig. 2. Filled symbols represent means with S.E. Curves of wild type NR1 (dashed line) and NR1-PKA mutant (thin line) receptors are redrawn from Fig. 2 for comparison.

FIG. 3.
Propagation—A second long-standing challenge to interpreting mutagenesis data has been to exclude the possibility that a mutation exerts its effect by introducing a structural defect which propagates to induce a conformational change in a remote part of the protein structure. It is possible that the change in apparent affinity ensuing from mutation of Ala-714 was the result of altered protein structure. In this case, modification of a distant residue could have induced a change in binding site affinity. The remaining experiments were designed to address this question.

We generated a mutation complementary to NR1-PKA, in which the targeted serine residue was incorporated into an N-linked glycosylation consensus site (13). Western analysis indicated that this mutant, NR1-G3, produced an increase in apparent molecular weight consistent with extracellular localization (Fig. 5). Its disruption of glycine affinity is approximately equal to a combination of point mutations at the individual residues that comprise it (Y711N, E712A, respectively, Table I). It is surprising that the resulting affinities and the properties of the substituted amino acids. Of the parameters investigated (including alpha-helix and beta-strand propensity, hydrophobicity, and frequency of occurrence in defined protein structures), the only amino acids not well fit by the volume regression model may offer a positive correlate to glycine affinity identified was side chain.

The correlation between side chain volume and affinity implies that the natural alanine residue at this position plays the architectural role of generating space for the ligand glycine. It furthermore infers that voluminous substitutions at this position create a steric hindrance that inhibits binding. Amino acids not well fit by the volume regression model may offer additional clues. Notably, the threonine mutation is the least well fit by this model. The increase in glycine affinity of the A714T mutation could be explained if the hydroxyl group of the
threonine was capable of substituting for a water molecule otherwise neighboring the glycine molecule. Along these lines, it is interesting to note that the volume correlation neatly divides the hydrophilic amino acids from the more hydrophobic. The two aromatic residues also impart less disturbance than their volumes would predict. This may be a product of their unique geometry or the particular affinity that glycine possesses for aromatics (8).

An alternate explanation of volume correlation would propose that larger amino acids exhibit greater disruption of proper protein folding and that the consequence of this disruption is decreased glycine affinity. The A714C mutation presented an opportunity to test this alternate interpretation. Cysteine is similar in size to alanine, and the A714C mutation exhibits a glycine affinity similar to that of the wild type receptor (Table I). Therefore, it can be assumed that little disruption of protein folding is generated by the A714C mutation. We utilized the unique reactivity of the cysteine residue to demonstrate the importance of position 714 in NMDA receptor function (Fig. 7).

Application of the thiol selective reagent dithionitrobenzoic acid (DTNB) to the A714C mutation greatly inhibited the function of the receptor (95% inhibition in 1 min). This oxidizing agent forms a mixed disulfide with free thiols. The mixed disulfide may remain or proceed to formation of a cysteine-cysteine bond in some instances (31, 32). Because of a slow reversibility of the inhibition incurred by A714C upon DTNB exposure, we were not able to accurately assess the glycine affinity of the DTNB-labeled mutant. The reversibility may be a result of thiol-disulfide interchange (33, 34).

DTNB was therefore applied in the presence of either L-glutamate or glycine. Protection from DTNB inhibition was afforded by glycine in a time-dependent manner. Co-application of L-glutamate but not L-glutamate argued against the possibility that the changes in glycine affinity arise from global changes in protein folding. Rather, it suggests that the presence of glycine protects A714C from reaction with DTNB by limiting the access of DTNB to the glycine binding pocket of the NMDA receptor. Probing of a ligand binding site with cysteine substitutions has been performed previously (35, 36). Limited protection from a cysteine labeling reaction has also been demonstrated (35). However, this experiment offers a
unique view of the time course of protection by the endogenous agonist from the reaction of a residue in a ligand binding site.

DISCUSSION

Our previous results indicated that both extremes of the M3-M4 linker of the NMDA receptor NR1 subunit exist in the extracellular space. This supported the hypothesized existence of a conserved hairpin pore structure similar to that found in voltage-gated K⁺ channels (13, 37). Subsequent evidence suggests that four other sites of the M3-M4 linker of NR1 are extracellular (14). In our current study, three separate measures provide further support to the three-transmembrane domain model. The shifts in glycine affinity, the increase in apparent molecular weight of an N-linked glycosylation site, and the unique susceptibility of a cysteine residue, each arising from mutations in the central portion of the M3-M4 linker, are all consistent with extracellular localization.

The significance of the M3-M4 linker in ionotropic glutamate receptor function has been recognized in a number of previous investigations (38–42). In fact, a segment of the M3-M4 linker has been assigned as the second of two lobes (S1 and S2) required for agonist binding, based on the homology between glutamate receptors and bacterial periplasmic binding proteins (8, 14, 15, 43–45). An important consequence of our findings is that the designation of position 714 as part of the glycine binding pocket is not predicted by the recognized homology between glutamate receptors and the bacterial periplasmic binding proteins (46).

This homology was used as a framework for the mutagenesis study that first revealed a contribution of the M3-M4 linker of the NMDA receptor to apparent glycine affinity (8). The authors superimposed the NR1 sequence onto the known three-dimensional structure of a member of the class of bacterial proteins (the lysine/arginine/ornithine binding protein or LAOBP) to generate a structural model of the glycine binding site of the NMDA receptor. The model was recently refined when several other residues in the M3-M4 linker were identified that affect apparent glycine affinity (14).

A detailed three-dimensional prediction of the structure of the glutamate binding site of non-NMDA receptors was recently produced (44). This model was also based upon the known LAOBP structure. Hence, the same bacterial periplasmic binding protein homology has been used to make structural predictions about L-glutamate binding in non-NMDA receptors (15, 43–45) and glycine binding in the NMDA receptor (8, 14). However, the binding site of the LAOBP protein accommodates only a single amino acid (46), while the NMDA receptor requires binding of two different amino acids for activation.

Mutations in analogous amino-terminal S1 residues affected GluR1 L-glutamate affinity and NR1 glycine affinity similarly (8, 47). Several of the NMDA receptor S1 mutations producing large changes in glycine affinity also affected L-glutamate affinity. Thus, it has been argued that this homologous binding site is conserved among all ionotropic glutamate receptors (43). Furthermore, it has been suggested that a lack of amino acid selectivity from the homologous binding region would be expected due to the nonselective nature of the bacterial proteins (43).

Several lines of evidence argue that L-glutamate and glycine do not possess the same binding domain within the NMDA receptor. Both molecules are necessary to activate the receptor (3, 4). Second, selective agonists and antagonists exist for each site (48). Finally, although the sites are allosterically coupled (5, 6), our own results and those of others (8) demonstrate that it is possible to affect the apparent affinity of one of the agonists without affecting the other.

There is evidence that two glycine molecules are necessary for activation of the native NMDA receptor complex (49). Whether these ligands interact at two distinct molecular sites remains unknown. Dramatic shifts in the apparent affinity for glycine have been produced by mutations in the S1 segment of the NMDA receptor (8, 50). The influence that position 714 exerts on glycine affinity may involve a second binding site or it may arise from interactions between the S1 and S2 segments.

It is curious to note the lack of influence on glycine affinity imparted by amino acid charge at this position (see Fig. 6). Therefore, it is likely that position 714 does not directly contribute a contact point for glycine binding. Rather, the volume correlation suggests that large amino acids at position 714 obstruct the access of the ligand to its binding site through steric hindrance. Alternatively, Ala-714 could reside in a region that undergoes a conformational change following glycine binding. If the NMDA receptor undergoes a hinge-bending motion upon ligand binding similar to that proposed for the bacterial periplasmic binding proteins (46), then a large portion of the protein could become protected when the two lobes close. Such a mechanism could possibly explain ligand-induced protection from DTNB modification.

Furthermore, the failure to consistently align this region of the glutamate receptor subunits with LAOBP is evidence for the lack of homology (Fig. 1). Furthermore, neither of the structural models of the NMDA receptor predicts any proximity of position 714 to the glycine binding site (8, 14). Recently, mutation of another amino acid in the M3-M4 of the NR1 subunit has been found to impart large shifts in glycine affinity (51). This residue is not predicted to contribute to glycine binding by the structural models either. These structural models offer insufficient detail to identify the precise location of Ala-714. Inspection of the diagram in Fig. 4 of Hirai et al. (14) suggests, however, that position 714 is located in a beta-sheet domain quite removed from the predicted glycine contact points.

Our results define position 714 as part of the glycine binding pocket. Since the region of the NMDA receptor that comprises the glycine binding pocket is distinct from the L-glutamate binding site, models based on bacterial protein homology cannot be expected to simultaneously predict both sites. Thus, although the recognized homology between ionotropic glutamate receptors and bacterial periplasmic binding proteins has contributed significantly to the current model of glutamate receptor structure, it may not serve to fully predict the complex nature of the NMDA receptor.

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