Expression and characterization of a glycine-binding fragment of the *N*-methyl-D-aspartate receptor subunit NR1

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N-Methyl-D-aspartate receptor channels are composed of an NR1 subunit and at least one of the NR2 subunits (NR2A–D). Activation of the *N*-methyl-D-aspartate receptor requires the co-agonists glycine and glutamate. It has been proposed that the NR1 subunit possesses a glycine-binding site. We have expressed a soluble form of the NR1 subunit, which was produced by connecting the N-terminal extracellular region with the extracellular loop between the third and fourth membrane segments, by a baculovirus system along with full-length and truncated membrane-bound forms. The soluble NR1 receptor was efficiently secreted into the culture medium and showed a high affinity for ligands. The K_d of a glycine-site antagonist, [³H]MDL 105,519 [(*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid], for the soluble receptor was 3.89 ± 0.97 nM, which was comparable to the K_d of 4.47 ± 1.39 nM for the membrane-bound full-length form. These values were close to the values reported previously with the use

of rat brain membranes and Chinese hamster ovary cells expressing the full-length form of the NR1 subunit. The K_i values of other glycine-site antagonists, L-689,560 (*trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline), 5,7-dichlorokynurenate and 5,7-dinitroquinoxaline-2,3-dione, for the soluble receptor were also similar to those for the full-length form of NR1. [³H]MDL 105,519 binding was also inhibited by the agonists glycine and D-serine. Thus the affinity and selectivity of ligand-binding characteristics of the NR1 subunit is conferred on the soluble form of the NR1 subunit. This soluble receptor provides a good experimental tool for initiating a biophysical analysis of the *N*-methyl-D-aspartate receptor channel protein.

Key words: baculovirus, glutamate receptor, ligand binding, ligand-gated ion channel, MDL 105,519.

INTRODUCTION

Glutamate receptors have pivotal roles in synaptic neurotransmission in vertebrate central nervous system [1]. Glutamate receptors are classified into two distinct classes: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs) [2,3]. The iGluRs consist of *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. Non-NMDA receptors are further subdivided into two groups: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors and kainate receptors. iGluRs are ligand-gated ion channels that transduce glutamate binding into cation influx. Activation of the NMDA receptor channel by glutamate opens a cation-selective ion channel when Mg^{2+} blocking is released by membrane depolarization. Furthermore, the NMDA receptor activity is modulated by polyamines, redox state and Zn^{2+} . The NMDA receptor is unique in its requirement for a co-activator, glycine [4]. The NMDA receptors are involved in several important physiological and pathological processes that include not only long-term potentiation and depression but also the progression of brain ischaemic damage and neural apoptosis.

NMDA receptor subunit NR1 is able to form a homomeric channel in *Xenopus* oocytes, although the channel activity is small. This property made it possible to clone the NR1 cDNA by the oocyte expression system [5]. In succession, four NR2 subunits (NR2A–D) were identified [6–8]. The precise subunit stoichiometry of the NMDA receptor channel *in vivo* has not been

determined. Recently, tetramer formation of the NMDA receptor has been proposed by a co-expression study of the wild-type subunit NR1 and a low-affinity mutant receptor of NR2 [9]. NR1 seems to be a key subunit for the relevant channel formation. Several studies have suggested that the binding of glutamate to the NMDA receptor requires NR1 and at least one of the NR2 subunits [2,3]. Glycine acts as a co-agonist and potentiates glutamate binding. The NMDA receptor is different in membrane topology from other neurotransmitter receptors for acetylcholine, γ -aminobutyric acid and glycine. A glycosylation-site tagging experiment [10] and an epitope protection assay [11] have elucidated the topology of a large N-terminal extracellular region, four hydrophobic segments (M1–M4), an extracellular loop region between M3 and M4, and a large cytoplasmic region. It has also been found that M2 forms the channel pore region. Furthermore, the iGluR subunits have two distinct regions whose amino acid sequences are similar to those of bacterial periplasmic binding proteins [12]. A leucine/isoleucine/valine-binding protein (LIVBP)-like region constitutes the first approx. 370 residues in the NR1 subunit. The similarity to lysine/arginine/ornithine-binding protein (LAOBP) is split between an N-terminal extracellular region (S1 region) proximal to M1 and the N-terminal portion (S2 region) of the extracellular loop between M3 and M4. Thus the LAOBP-like region is composed of the S1 and the S2 regions separated by the first three membrane segments [13]. A glycine-binding site has been assigned to the LAOBP-like region in the NR1 subunit [14,15]. A glutamate-

Abbreviations used: DCKA, 5,7-dichlorokynurenate; (+)-HA-966, (R)(+)-3-amino-1-hydroxypyrrolidin-2-one; iGluR, ionotropic glutamate receptor; L-689,560, *trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline; LAOBP, lysine/arginine/ornithine-binding protein; LIVBP, leucine/isoleucine/valine-binding protein; MDL 105,519, (*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid; mGluR, metabotropic glutamate receptor; MNQX, 5,7-dinitroquinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; PNGase F, peptide N-glycosidase F.

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binding site has been proposed at the corresponding region of the NR2 subunits [16,17].

We have recently reported the formation of a soluble and active form of mGluR subtype 1 [18] that consists mainly of an LIVBP-like region. However, neither the function of the LIVBP-like region in the NMDA receptor nor the precise boundary between the LIVBP-like region and the S1 region has not yet been determined. We have tried to obtain a soluble receptor consisting of the whole N-terminal extracellular region connected with the entire loop region between M3 and M4. This soluble receptor has showed ligand-binding capacity comparable to that of the full-length receptor. Ligand-binding affinities of the expressed receptors to several agonists and antagonists have been examined.

EXPERIMENTAL

Reagents

All chemicals were of analytical grade. [³H]MDL 105,519 [(*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid] (2.66 TBq/mmol; 72.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). The anti-NR1 monoclonal antibody MAB363 was obtained from Chemicon International (Temecula, CA, U.S.A.). The second goat anti-(mouse IgG) was from Promega (Madison, WI, U.S.A.). Glycine, 5,7-dichlorokynurenate (DCKA), 5,7-dinitroquinoxaline-2,3-dione (MNQX), (*R*)(+)-3-amino-1-hydroxypyrrrolidin-2-one [(+)-HA-966], *trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline (L-689,560) and D-serine were purchased from Tocris Cookson (Langford, Bristol, U.K.). MDL 105,519 was obtained from RBI (Natick, MA, U.S.A.). L-Glutamate was purchased from Wako Pure Chemical Industries (Osaka, Japan). Details of insect cells and culture medium ingredients were as described previously [18].

Construction of the transfer vectors and the recombinant baculoviruses

For the construction of pNR1-dM4CR, PCRs were performed with primer F (5'-GTATCCTAGGCATGGTGTGGG-3') and each of primer R1 (5'-GGATCCTCATCAGTGATGATGATGATGATGCTCAAAAGTGAGGGTTGCAGAG-3') and primer R2 (5'-GGATCCTCATCATAGATCCTCTCTGAG-ATGAGCTTTTGCTCCTCAAAAGTGAGGGTTGCAGAG-3'), with pN60 [5] as a template. *BlnI*-*Bam*HI fragments (1.0 kbp) cut out of the PCR products and a 1.9 kbp *SacI*-*BlnI* fragment of pN60 were ligated into *SacI*/*Bam*HI-digested pUC118, resulting in pUC118NR1his and pUC118NR1myc respectively. A 1.0 kbp *EcoRI*-*SacII* fragment and a 1.6 kbp *SacII*-*Bam*HI fragment of pUC118NR1myc were ligated into pVL1392 (PharMingen, San Diego, CA, U.S.A.).

For pNR1-F1, a 1.0 kbp *SacI*-*SacII* fragment of pN60 and a 1.6 kbp *SacII*-*Bam*HI fragment of pUC118NR1myc were ligated into *SacI*/*Bam*HI-digested LITMUS[™] 28 (New England Biolabs, Beverly, MA, U.S.A.), resulting in LITMUS28NR1myc. PCR was performed with primer 8 (5'-ATCGGCATGCGCAAGGA-3') and primer 9 (5'-GGATCCGATATCTCATCATAGATCC-TCCTCTGAGATGAGCTTTTGCTCGCTCTCCCTATG-ACGGG-3'), with pN60 as a template. The PCR product was digested with *SphI*/*Bam*HI and ligated into the identical restriction enzyme sites of LITMUS28NR1myc, resulting in LITMUS28NR1myc. A 2.8 kbp *StuI*-*Bam*HI fragment of LITMUS28NR1myc was ligated into the filled-up *Bgl*II and *Bam*HI sites of pVL1392.

For pNR1-L1, PCR was performed with primer 4 (5'-CAGAGAAACCTCGAGGATACC-3') and primer 5 (5'-AAATCCCTCTTCTTCGGCATTGACCTCACCTCGGTGCA-TGTGCTCTGAAAAGGCTGCATAAATG-3'), with pUC-118NR1myc as a template. Another PCR was performed with primer 6 (5'-TCGACCGAGGGTGAGGTCAATGCC-GAAGAAGAGGGATTAACTTGGCAGCTTTCTCTG-TTG-3') and primer 7 (5'-CCTCCGGAAGTAGATGTCC-3'). The PCR products were mixed and the second PCR was performed with primers 4 and 7. A 0.4 kbp *XhoI*/*Acc*III-digested PCR product was ligated into the *XhoI* and *Acc*III sites of pUC118NR1his (described above in the construction of pNR1-dM4CR), resulting in pUC118NR1L1his. A 0.9 kbp *EcoRI*-*SacII* fragment and a 1.3 kbp *SacII*-*Bam*HI fragment of pUC118NR1L1his were ligated with *EcoRI*/*Bam*HI-digested pVL1392. All the PCR products were fully sequenced on both strands. These transfer vectors were used for transfection to insect cells with Baculogold[™] (PharMingen) and the recombinant viruses were obtained. Details for recombinant baculovirus purification, amplification and infection into insect cells have been described in [18].

Membrane preparation

Membranes of the insect cells were prepared by nitrogen cavitation as described in [18] and stored at -80 °C until use.

Immunoblotting

Cell membrane and culture medium were separated by SDS/PAGE and electroblotted to a nitrocellulose membrane (Schleicher & Schull). Recombinant NR1 proteins were identified by using the monoclonal antibody MAB363 and anti-(mouse IgG)-conjugated alkaline phosphatase. Colour development was done by a commercial detection kit (Promega).

Ligand binding assay and data analysis

Membrane fractions of baculovirus-infected cells were incubated with [³H]MDL 105,519 in a total volume of 250 µl of buffer A (50 mM Hepes/NaOH, pH 7.7) at 4 °C for 1 h. The reaction mixtures were aspirated on GF/C filters (Millipore Corp., Bedford, MA, U.S.A.). After being washed with buffer A and dried, the radioactivity remaining on the filters was counted by scintillation counting. Non-specific binding was defined in the presence of 100 mM glycine. For the soluble NR1-L1 secreted into culture medium, the ligand-receptor complex was precipitated on ice with 15% (w/v) 6 kDa poly(ethylene glycol) solution containing 1 mg/ml γ-globulin; it was then pelleted by centrifugation at 14000 *g* for 15 min. The precipitated material was dissolved in 1 ml of water and the radioactivity was counted in the scintillation counter. Data analysis was performed with PRISM II (Graphpad Software, San Diego, CA, U.S.A.). Saturation binding curves were fitted to a one-site binding model and the *K_d* and *B_{max}* values were calculated. The errors of the *K_d*, *B_{max}* and IC₅₀ values are S.E.M. Dose-response curves were fitted to sigmoidal curves.

Deglycosylation of the recombinant NR1 glutamate receptors

Membrane fractions and supernatants of cells infected with baculoviruses were denatured by boiling with 0.1% SDS for 2 min; the reaction buffer was then adjusted to 20 mM sodium phosphate buffer, pH 7.0, containing 50 mM EDTA, 10 mM NaN₃ and 0.5% (v/v) Nonidet P40, followed by boiling for 2 min. Half of the reaction mixture was incubated for 24 h with

0.2 unit of peptide N-glycosidase F (PNGase F; Boehringer Mannheim, Mannheim, Germany). One unit is the enzyme activity that hydrolyses 1 nmol of dansyl fetuin glycopeptide within 1 min at 37 °C. The remaining half was not treated with enzyme and was used as a control. Both mixtures were subjected in parallel to SDS/PAGE [7.5% (w/v) gel] followed by immunoblotting.

RESULTS

Figure 1 is a schematic representation of the transfer vectors used in the baculovirus infection experiments. pNR1-F1 encoded a full-length membrane-bound form of NR1 subunit. pNR1-dM4CR was designed to produce a truncated NR1 lacking M4 and the entire cytoplasmic region. pNR1-L1 was expected to yield a soluble form of NR1 by connecting the N-terminal extracellular region and the extracellular loop between M3 and M4 with a 13-residue linker peptide [19]. pNR1-F1 and pNR1-dM4CR were flanked by a c-Myc tag, C-terminally. pNR1-L1 was flanked by six histidine codons. These three transfer vectors contained the original signal sequence of NR1. The expressed proteins were designated NR1-F1, NR1-dM4CR and NR1-L1.

Each recombinant baculovirus for the expression of the full-length (NR1-F1), truncated (NR1-dM4CR) and soluble (NR1-L1) forms was plaque-purified and used to infect Sf9 (membrane-bound forms) and High Five insect cells (a soluble form). An immunoblotting analysis of the expressed NR1s is shown in Figure 2(A). Membrane fractions prepared from the insect cells infected with the recombinant viruses for NR1-F1 and NR1-dM4CR yielded two bands. Because the faster-migrating bands in lanes 1 and 2 corresponded to the size of the unglycosylated forms, the upper slowly migrating bands seemed to be the glycosylated forms. The culture medium of the insect cells infected with the recombinant virus for the soluble form, NR1-L1, contained a 105 kDa polypeptide detected by the anti-(NR1 subunit) monoclonal antibody (Figure 2A, lane 3). As the molecular mass calculated from the amino acid sequence was 81.8 kDa, the 105 kDa band seemed to be a glycosylated form. Neither the membrane fraction (Figure 2A, lane 4) nor the culture medium (lane 5) of insect cells infected with the wild-type

Figure 1 Schematic diagram of recombinant transfer vectors for NR1s in insect cells

pNR1-F1 encodes the full-length NR1 subunit; pNR1-dM4CR terminates before M4; pNR1-L1 encodes the N-terminal extracellular region and the extracellular loop region. The two regions are connected by a 13-residue linker peptide. pNR1-F1 and pNR1-dM4CR are flanked by a c-Myc tag that encodes the c-Myc epitope. pNR1-L1 is flanked by a His tag that contains six consecutive histidine codons. Abbreviation: aa, amino acid residues.

Figure 2 Immunoblot showing the expression of the NR1s produced by baculovirus-infected insect cells (A) and glycosidase treatment of the expressed NR1s (B)

(A) Membrane fractions (20 μ g) of Sf9 cells infected with the recombinant viruses for NR1-F1, NR1-dM4CR and the wild-type virus (AcNPV) and the supernatant (15 μ l) of High Five[®] insect cells infected with the recombinant viruses for NR1-L1 and AcNPV were subjected to SDS/PAGE [7.5% (w/v) gel]. Proteins were transferred to a nitrocellulose membrane and probed with the anti-NR1 monoclonal antibody. The positions of molecular mass standards are indicated at the left. (B) Aliquots (6 μ g) of samples used in (A) were denatured with 0.1% SDS and incubated with 0.2 unit of PNGase F in a total volume of 100 μ l of glycosidase buffer described in the Experimental section. Digestion was performed at 37 °C for 24 h. Digested samples (+) (lanes 1, 3 and 5) and control samples (—) (lanes 2, 4 and 6) incubated without enzyme were subjected to parallel SDS/PAGE [7.5% (w/v) gel] followed by immunoblotting.

virus showed any signal. Figure 2(B) shows the result of a deglycosylation experiment. The slower-migrating forms of NR1-F1 (Figure 2B, lane 2) and NR1-dM4CR (lane 4) were digested with PNGase F (lanes 1 and 3). The glycosidase treatment resulted in a decrease in the molecular mass of NR1-L1 (Figure 2B, lane 5). The faster-migrating bands observed in Figure 2(B) (lanes 3–6) might have been degradation products formed during the reaction. Thus the glycosylation of the expressed NR1s was confirmed.

Next the ligand-binding capacities of the recombinant NR1s were examined. The full-length form, NR1-F1, showed a specific binding of [³H]MDL 105,519, a glycine-site antagonist [20]. Its

Figure 3 Saturation isotherms (left panels) and Scatchard plots (right panels) of the binding of [3 H]MDL 105,519 to the NR1s produced by baculovirus-infected insect cells

Details are described in the Experimental section. In brief, membrane fractions (45 μ g) of Sf9 cells infected with the recombinant viruses for NR1-L1 (A) and NR1-dM4CR (B), and 50-fold concentrated supernatant (30 μ l) of High FiveTM insect cells infected with the recombinant viruses for NR1-L1 (C) were incubated in a binding buffer (50 mM Hepes, pH 7.7) with different concentrations of [3 H]MDL 105,519 at 4 $^{\circ}$ C for 1 h. Non-specific binding (□) was determined in the presence of 100 mM glycine and was subtracted from total binding (■) for the determination of specific binding (●). The results were analysed by PRISM II for saturation kinetics (left panels) and for Scatchard analysis (right panels). A representative result from three independent experiments is shown. Each binding was performed in triplicate and is shown as the mean $\pm$ S.D. A non-linear regression analysis of NR1-F1 revealed a K_d of 4.47 ± 1.39 nM and a B_{max} of 4.748 ± 0.822 pmol/mg of protein; NR1-dM4CR gave rise to a K_d of 2.83 ± 0.25 nM and a B_{max} of 0.997 ± 0.119 pmol/mg of protein; NR1-L1 showed a K_d of 3.89 ± 0.97 nM and a B_{max} value of 2.767 ± 0.398 pmol/mg of protein. These K_d and B_{max} values are means $\pm$ S.E.M.

binding was displaced by excess unlabelled glycine and was saturable, as shown in Figure 3(A). Non-linear regression analysis yielded a K_d of 4.47 ± 1.39 nM and a B_{max} of 4.748 ± 0.822 pmol/mg of protein ($n = 3$). Scatchard transformations are shown in Figure 3 (right panels). The truncated form, NR1-dM4CR, also showed saturable binding (Figure 3B). A comparable K_d of 2.83 ± 0.25 nM was obtained. Next the culture medium of the insect cells infected with the recombinant virus for the soluble form, NR1-L1, was concentrated and dialysed against the binding buffer. This preparation showed a ligand-binding capacity. Its K_d of 3.89 ± 0.97 nM (Figure 3C) was consistent with the value obtained for the membrane-bound full-length form, NR1-F1.

The inhibition of binding of [3 H]MDL 105,519 to the full-length NR1-F1 (Figure 4A) and the soluble NR1-L1 (Figure 4B) with several unlabelled agonists and antagonists was examined.

The IC_{50} values of MDL 105,519 for NR1-F1 and NR1-L1 were 4.0 ± 1.2 and 4.9 ± 1.2 nM respectively. Calculated K_i values of the antagonists and agonists for NR-F1 and NR1-L1 are shown in Table 1. The K_i values of the ligands for NR-L1 were comparable to those for NR-F1. L-Glutamate did not show any inhibition up to 100 μ M. The K_i values of the antagonists MDL 105,519 and DCKA for NR1-L1 were close to those obtained for rat brain membrane [20]. An antagonist L-689,560 showed a K_i value comparable to that of MDL 105,519. The K_i values of the agonists glycine and D-serine for NR1-F1 and NR1-L1 were an order of magnitude smaller than those obtained for rat brain membrane preparations but comparable to those of the recombinant NR1 expressed in Chinese hamster ovary cells [21]. Thus the soluble form of the NR1 subunit, NR1-L1, gave values very similar to those obtained for the membrane-bound form, NR1-F1.

Figure 4 Dose-response curves of various antagonists and agonists in inhibiting the binding of [3 H]MDL 105,519 to NR-F1 and NR-L1

Indicated concentrations of antagonists with the addition of [3 H]MDL 105,519 (1 nM), were incubated with 45 μ g of membrane fraction of Sf9 cells infected with the recombinant virus for NR1-F1 (A) or with 30 μ l of the 50-fold concentrated supernatant of High FiveTM cells infected with the recombinant virus for NR1-L1 (B). Each point shows the mean $\pm$ S.D. of a representative one of five (A) and three (B) experiments done in triplicate. The displacement curves were obtained by sigmoidal fitting with PRISM II. Symbols: $\blacksquare$, glycine; $\blacktriangle$, L-glutamate; $\blacktriangledown$, DCKA; $\blacklozenge$, MDL 105,519; $\bullet$, MNQX; $\square$, (+)-HA-966; $\triangle$, L-689,560; ∇ , D-serine.

DISCUSSION

Amino acid sequences of glutamate receptors show similarity to those of bacterial amino acid binding proteins. Dozens of tertiary structures of the bacterial binding proteins have been determined by crystallography. They consist of two similar globular domains; their ligand-binding sites are located deep in the cleft between the two domains [22]. On the basis of ligand-bound and ligand-unbound crystal structures and light-scattering studies, the Venus fly-trap model for ligand binding has been probed: the binding of ligand to one domain induces a bending motion at the hinge region and causes the other domain to participate in the binding. Whether analogous ligand binding and domain movement occurs in glutamate receptors is an intriguing and important question. Recently, Armstrong et al. [23] reported the crystal structure of an S1-S2 fusion protein derived from GluR2, one of the non-

Table 1 Affinity of glycine-site ligands for wild-type NR1-F1 and soluble-type NR1-L1

The K_i values for inhibition by various compounds of [3 H]MDL 105,519 binding in membranes from the cells infected with recombinant virus for NR1-F1 and in culture supernatant of the cells infected with recombinant virus for NR1-L1 were measured. The K_i values (means $\pm$ S.E.M.) ($n = 5$ for NR1-F1; $n = 3$ for NR1-L1) were calculated from the K_d values in Figure 3, with the use of the IC_{50} values determined for the ligands (Figure 4) by the equation $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ and K_d are the concentration and dissociation constant of the radioligand respectively. Abbreviation: n.d., not determined.

Compound	K_i (μ M)	
	NR1-F1	NR1-L1
MDL 105,519	0.0032 ± 0.0004	0.0039 ± 0.0010
L-689,560	0.00594 ± 0.00061	0.00594 ± 0.00144
DCKA	0.254 ± 0.059	0.554 ± 0.105
MNQX	0.498 ± 0.052	0.456 ± 0.0357
(+)-HA-966	262.26 ± 74.14	162.58 ± 35.61
Glycine	6.34 ± 0.60	13.82 ± 0.19
D-Serine	2.19 ± 0.35	4.95 ± 0.63
L-Glutamate	n.d.	n.d.

NMDA receptors, in a complex with its ligand, kainate. They concluded that the binding of the ligand induced an intermediate degree of domain closure relative to a bacterial amino-acid-binding protein.

In the present study we have succeeded in obtaining a soluble and active fragment of NR1. The full-length membrane-bound form of NR1 has been expressed and shown to have the ability to bind ligands. Before making the soluble form, we attempted to make a truncated receptor, NR1-dM4CR, encoding the amino acid sequence that starts at the native N-terminus and terminates at the end of the extracellular loop between M3 and M4. The ligand-binding capacity of this truncated receptor was similar to that of the full-length form. This result shows that the extracellular loop region is correctly folded even when M4 and the entire cytoplasmic region has been deleted. Sutcliffe et al. [24] have speculated that M4 in iGluRs is positioned away from the channel pore but interacts with M1 and M3. Because [3 H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (MK801), a channel blocker, did not bind to NR1-dM4CR (results not shown), M4 might be necessary for channel formation. Next, we also deleted all the membrane regions and the cytoplasmic region. The resultant soluble receptor, NR1-L1, was successfully secreted into the culture medium and showed a remarkable affinity for the ligands. The K_d of the glycine-site antagonist [3 H]MDL 105,519 for NR1-L1 is comparable to that for the membrane-bound form, NR1-F1. The K_i values of other ligands for NR1-L1 are similar to those for NR1-F1. Thus we have isolated the ligand-binding region of the NR1 subunit as a soluble protein with ligand-binding affinity and selectivity similar to those of the membrane bound form.

Recently, Ivanovic et al. [25] reported similar results that a polypeptide fragment containing only the S1 and the S2 regions of the NR1 subunit is able to form a glycine-binding site. In comparison with our soluble receptor, their fragment was devoid of the N-terminal LIVBP-like region preceding the S1 region and the N-terminal portion (seven residues) of the extracellular loop region between M3 and M4. Their measured K_d for MDL 105,519 binding, 5.22 ± 0.13 nM, was close to our K_d of 3.89 ± 0.97 nM. Therefore the glycine-binding region can be further localized within the S1 and the S2 regions. The LIVBP-

like region seems to perform no inhibitory or enhancing effects on glycine binding.

It has not been determined whether the LIVBP-like region forms the orifice of the channel or locates in a remote position from the inner wall of the NMDA receptor channel. Interestingly, an LIVBP-like region is found in the extracellular region of mGluR. This region functions as a ligand-binding domain in mGluR. Thus the LIVBP-like region is conserved in two different receptor proteins. Although ligand binding has not yet been assigned to the LIVBP-like region of the NMDA receptor, it is an attractive idea that some ligand molecule might bind to the LIVBP-like region and modify the signal transmission allosterically. Our expressed soluble molecule might provide an experimental device for elucidating the cryptic function of the region.

In bacterial binding proteins, ligand-unloaded open and ligand-loaded closed forms have been characterized. If two configurations that correspond to those of the bacterial binding proteins exist in the NMDA receptor, it remains to be elucidated whether our expressed NMDA receptor fragment represents an open or a closed form. In comparison with rat brain membrane preparations, the recombinant NR1-F1 and NR1-L1 show a lower affinity for the agonists glycine and D-serine but not for antagonists. The S1–S2 fusion protein did not show a high affinity for the agonists either [25]. It is reported that antagonist binding is less affected by amino acid mutagenesis in the S2 region, whereas agonist binding is affected by amino acid mutation both in S1 and S2 [14,15]. Thus the participation of both S1 and S2 regions possibly provided by a closed form seems to be predictable for agonist binding. Because our NR1 subunits show a decreased affinity for agonists, the homomeric NR1 proteins might exist in an open form, namely an antagonist-preferring form as postulated [25,26]. Co-expression with NR2 subunits is expected to improve the affinity for agonist. Even the heteromeric receptor complexes expressed in yeast, however, have not shown displacement of [³H]MDL 105,519 by 100 μ M glycine or D-serine [27]. Therefore more extensive expression studies of agonist binding are needed.

In spite of many experiments, the molecular mechanism of NMDA receptor channel opening or closing has not been clarified owing to the difficulty of reconstituting the channel by using pure receptor material. The concentration of the co-agonist glycine in the synaptic space seems to be high enough for the channel protein to be already occupied by glycine in the basal state [28]. How the glutamate binding signal is transmitted in NMDA receptor molecules and transduced to channel opening or closing is totally unknown. We speculate that the conformational change of bacterial amino-acid-binding protein-like domains induced by the binding of glutamate triggers a spatial rearrangement of the

S1 and the S2 regions. An appropriate conformation, tuned only when complex requirements are satisfied, might cause the change in orientation of membrane segments and gating of the channel.

We thank Dr. Yoshiro Shimura for helpful suggestions and continuous encouragement.

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Received 17 December 1998/8 March 1999; accepted 30 March 1999