Modulation by L- and D-Isoforms of Amino Acids of the L-Glutamate Response of *N*-Methyl-D-aspartate Receptors

Kazumi Sakata,[‡] Teruyuki Fukushima,[‡] Liu Minje,[‡] Tarou Ogurusu,[‡] Hideharu Taira,[§] Masayoshi Mishina,[||] and Ryuzo Shingai*,[‡]

Department of Computer and Information Science, Faculty of Engineering, Iwate University, 4-3-5 Ueda, Morioka, Iwate 020-8551, Japan, Department of Bioscience and Technology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan, Department of Molecular Neurobiology and Pharmacology, School of Medicine, University of Tokyo, Tokyo 113-0033, Japan, and CREST, Japan Science and Technology Corporation, Saitama 332-0012, Japan

Received February 2, 1999; Revised Manuscript Received May 5, 1999

ABSTRACT: *N*-Methyl-D-aspartate (NMDA) receptor subtypes $\epsilon 1$ and $\zeta 1$ were coexpressed in *Xenopus* oocytes for the investigation of the magnitude of augmentation of the L-glutamate response by 20 common L-amino acids and their 19 D-isoforms. Simultaneous application of L- and D-alanine, -cysteine, and -serine, or glycine and L-glutamate potentiated the glutamate-induced current. Other amino acids produced only marginal effects. Analysis of the relationship between the response and amino acid size revealed that the critical threshold size is between those of cysteine and aspartate. No amino acid alone induced a current. The effects of L- and D-alanine, -cysteine, and -serine applied with L-glutamate were concentration-dependent. Molecular modeling of these three amino acids revealed a positive relationship between the charge at an atom of the side chain and the receptor sensitivity, which may explain the efficacies of these amino acids.

N-Methyl-D-aspartate (NMDA) receptors have several modulation sites (*1*−*4*), among which the glycine-binding site is the major site involved in receptor excitation. Glycine, at nanomolar concentrations, increases considerably the probability of NMDA receptor channels opening (*5*, *6*), and many analogues interact with this glycine-binding site and activate the NMDA receptor.

An electrophysiological study of NMDA receptors in *Xenopus* oocytes into which messenger RNA from the rodent brain had been injected (*7*) and a binding study (*8*) revealed that serine and alanine could bind to the glycine-binding site of and regulate NMDA receptors and that the L-isomers of serine and alanine were less potent than their respective D-isomers by at least 1 order of magnitude. The 20 common amino acids in proteins, with the exception of glycine, exist in either of two stereoisomeric forms, the L- and D-forms. Serine, one of these common amino acids, is present in its D-form in human and rodent brains (*9*−*12*), modulates glutamatergic functions (*13*, *14*), and seems to be synthesized by brain synaptosomes (*15*).

Free D-serine potentiates the L-glutamate response of cloned NMDA receptors expressed in *Xenopus* oocytes (*16*). Several workers (*16*−*20*) have used this cloned NMDA receptor expression system to investigate the functional properties of pure subunit-specific forms. They compared the responses to glycine and D-serine of NMDA receptors with several subunit combinations and showed that the concentration of D-serine in the brain was high enough to activate the receptors.

The structure of the glycine-binding site was investigated by Kuryatov et al. (*21*), who recorded the electrophysiological responses of mutated NMDA receptors containing the rat NR1 subunit, which is homologous with the mouse $\zeta 1$ subunit, and proposed a detailed model of the glycine-binding site. They showed which amino acid residues were crucial for glycine binding by the receptor and proposed the three-dimensional arrangement of the binding site based on the structure of a homologous bacterial protein. Laube et al. (*22*) located the glycine- and glutamate-binding sites in the rat NR1 subunit and NR2 subunits, respectively, and devised a detailed model of the glutamate-binding site of the latter.

In this study, we examined the D- and L-isoforms of 19 common amino acids and determined the structures required for NMDA receptor activation and their basic electrophysiological properties. We also determined the structural features of the active amino acids by carrying out computer simulation. Although the structures of the ligands that were active at the NMDA receptor differed slightly, the sensitivities of this receptor to them differed considerably. Therefore, we carried out computer simulation to reveal slight structural differences among the ligands. The combination of electrophysiological experimentation and computer simulation enabled us to demonstrate the relationships between the structural features of the ligand molecules and the NMDA receptor sensitivities to them.

* To whom correspondence should be addressed: Department of Computer and Information Science, Faculty of Engineering, Iwate University, 4-3-5 Ueda, Morioka, Iwate 020-8551, Japan. Phone: +81-19-621-6479. Fax: +81-19-621-6487. E-mail: shingai@cis.iwate-u.ac.jp.

[‡] Faculty of Engineering, Iwate University.

[§] Faculty of Agriculture, Iwate University.

[||] University of Tokyo and CREST, Japan Science and Technology Corp.

10.1021/bi990246k CCC: $18.00
Published on Web 07/14/1999

MATERIALS AND METHODS

Electrophysiology of Receptors Expressed in Xenopus Oocytes. The ϵ1 and ζ1 subunit-specific cRNAs were synthesized in vitro using T3 RNA polymerase in the presence of cap dinucleotide 7mGpppG and plasmids carrying the cloned receptor cDNA cleaved with *Not*I. *Xenopus laevis* females (Hamamatsu Dobutsu Kyozai Co., Hamamatsu, Japan) were anesthetized deeply with 0.15% (w/v) tricaine methylsulfonate (MS222, Aldrich Chemical Co., Milwaukee, WI); an abdominal incision (1.5 cm long) was made, and the ovarian lobes were removed and placed in modified Barth's medium [MBM; 88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.5 mM sodium pyruvate, and 7.5 mM Tris (pH 7.6), supplemented with 40 units/mL penicillin and 1000 units/mL streptomycin]. The chemicals used to make up the MBM were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The incision was sutured and allowed to heal. The ovarian lobes in MBM were dissected into several pieces, and the oocytes were placed in fresh MBM and defolliculated manually with fine forceps without proteinase enzyme treatment. The defolliculated oocytes were incubated in MBM for 3−5 h at 18 °C to reveal injured oocytes, which were removed and discarded. Then, 40 nL of cRNA solution containing about 80 ng of RNA was injected into each intact oocyte, which was incubated in MBM at 18 °C for 48 h before electrophysiological recording. Under these conditions, the oocytes survived for about 6 days after the injection.

The oocytes were placed in a small recording chamber, superfused with Mg^{2+}-free Ringer solution [110.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, and 8.0 mM Tris (pH 7.4)] at 20−22 °C, impaled with microelectrodes filled with 3 M KCl, and voltage-clamped (CEZ1200, Nihon Kohden Co., Tokyo, Japan) at a holding potential of −60 mV between the two electrodes. The oocytes were superfused by gravity feed (4 mL/min), and amino acids dissolved in the superfusate were applied by means of three-way taps each with a dead time of 7 s. Current and voltage signals were recorded on a videotape recorder (Sony, Tokyo, Japan) via a PCM digitizer (VR-10B Instrutech Co.), and the replayed data were printed out on a chart recorder, or digitized and analyzed using a personal computer.

An agar bridge containing Mg^{2+}-free Ringer solution was used for the recording, because if the Ag−AgCl electrode was immersed directly in the recording chamber as the ground electrode, the amino acids introduced into the recording chamber interacted with the Ag−AgCl electrode, and generated large currents, depending on the current leakage of the oocytes that was recorded, as described previously (*23*, *24*). Therefore, the Ag−AgCl ground electrode was not used in this study, and an agar bridge containing Mg^{2+}-free Ringer solution was placed between the saline in the recording chamber and the Ag−AgCl electrode.

The efficacy of each amino acid was assessed by measuring the peak current induced after its application and comparing this current with the peak current induced after the application of 10 μM L-glutamate and 10 μM glycine. The chemicals used to make up the Ringer solutions and the amino acids were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All the other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of guaranteed reagent grade. Each amino acid (30 μM) and 10 μM L-glutamate were applied simultaneously for at least 1 min. For the D- and L-isoforms of alanine, cysteine, and serine, the ratios of the peak responses evoked by 10 μM L-glutamate and 10 μM amino acid to that evoked by 10 μM L-glutamate and 10 μM glycine were calculated. Fresh solutions of D- and L-cysteine were prepared from the powders each day and used that day. The cysteine solution was placed under a vacuum in an aspirated desiccator for 15 min before recording to remove the air in the solution and prevent oxidization of cysteine.

Determination of the Ligand Structure with the Lowest Energy. To determine the charge distribution within the ligand amino acids, computer simulation was performed in three steps. In the first step, the lowest-energy structure of each ligand molecule was determined using the program package TINKER (J. Ponder, Washington University School of Medicine, St. Louis, MO). Simulation was performed using an AT-compatible personal computer running under the Linux 2.0.33 operating system. The lowest-energy structures of glycine, alanine, β-fluoroalanine, cysteine, and serine were determined. As optical isomerism does not affect the results, the atomic coordinates of the L-isoforms of the amino acid molecules were generated. To establish the lowest-energy conformation of each amino acid, the energies at various torsion angles were calculated using the parameter set of TINKER. The conformations were generated from a set of torsion angles rotated in 10° increments, and then the energies were calculated. The environmental dielectric constant was set to 78.4 under the assumption that the ligand molecule existed in an aqueous environment. Under the conditions of the ligand solution at neutral pH, the proton of the carboxyl group was removed and the nitrogen of the amino group was set to be protonated. Therefore, the total charge within the amino acid was zero. Of the conformations determined using the various torsion angle sets for each amino acid ligand, the structure with the lowest energy was used as the initial structure for the next calculation step using MOPAC.

In the second step, the more stable structure was determined using WinMOPAC version 2 (Fujitsu Ltd.) running under the Microsoft Windows95 operating system. The dielectric constant of the environment was set to 78.4 to simulate an aqueous environment for the ligand molecule, and the calculation was performed using a PM3 Hamiltonian, under the assumption that all the torsion angles and the bond lengths were optimized. The lowest-energy structure determined in this step was then used as the initial structure for the next step, energy calculation of the ligand with two water molecules.

In the third step, to simulate the situation in which the ligand molecule forms hydrogen bonds with the amino acids of the receptor which are located in the binding site, two water molecules were placed as the virtual amino acid side chains of the receptor, based on the model of Laube et al. (*22*). The oxygen atom of one water molecule was 1.95 Å from the nitrogen atom of the amino group of the ligand. The hydrogen atom of the other water was 2.14 Å from the oxygen atom of the carboxyl group of the ligand.

The initial structures were formed with the ligand possessing the lowest-energy structure determined in the second step and two water molecules with a set of torsion angles relative to the ligand rotated in 20° increments. Then, the energy was minimized under the condition whereby all the torsion angles and bond lengths of the ligand, and the relative torsion angles of the water molecules, were optimized. The bond lengths and bond angles within the water, and the distances from the water molecules to the ligand, were not optimized. The charge distribution within the ligand molecule was obtained as the average of the lowest-energy structures.

RESULTS

Electrophysiological Responses to Various Amino Acids. The membrane currents generated by 38 amino acids were recorded to determine which amino acid molecules activated the NMDA receptors via interaction with its glycine-binding site. The peak currents induced in every cell by coapplication of 10 μM L-glutamate and 10 μM glycine were measured and taken as the standard response against which the peak responses induced by the other amino acids with and without L-glutamate were evaluated.

The ratio of the peak response of $\epsilon1\zeta1$ heterooligomeric receptors evoked by 10 μM L-glutamate and 30 μM L- or D-amino acid to the standard peak response evoked by 10 μM L-glutamate and 10 μM glycine was obtained for each of the 19 common L-form amino acids and their corresponding D-forms. The ratios obtained from the same oocytes were used to assess the efficacies of these 38 amino acids on NMDA receptor channels. None of the amino acids that were tested, except both the D- and L-forms of alanine, cysteine, and serine, exhibited efficacies comparable to that of glycine. The magnitudes of the currents evoked by the D- and L-forms of the inactive amino acids were only about 0.3−4.3% of the reference glycine-induced current. Both enantiomers of alanine, cysteine, and serine activated the receptor and evoked currents comparable to the glycine-induced reference current (Table 1). The receptors composed of $\epsilon2\zeta1$ or $\epsilon3\zeta1$, where $\epsilon2$ and $\epsilon3$ are highly homologous to the $\epsilon1$ subunit, also exhibited similar properties; only D- and L-forms of alanine, cysteine, and serine exhibited efficacies comparable to that of glycine (data not shown).

The ratios of the peak responses evoked by 10 μM L-glutamate and 10 μM amino acid to the standard response evoked by 10 μM L-glutamate and 10 μM glycine for these six active amino acids were also determined. For example, the currents evoked by D- and L-cysteine (10 μM) and 10 μM L-glutamate were 16.3 and 1% of the standard value, respectively. The other amino acids exhibited no or only marginal potentiating effects. The efficacies of these three D-amino acids were higher than or comparable to those of their corresponding L-isoforms. D-Alanine and D-serine exhibited potentiating potencies comparable to that of glycine, whereas D-cysteine potentiated the L-glutamate response to a lesser extent than glycine. As the side chain structure of threonine is similar to that of serine, i.e., a hydroxyl group, the efficacy of threonine was investigated by applying 10 μM L-glutamate with 100 and 1000 μM threonine to the receptors, but the activity of threonine was not comparable to that of glycine (data not shown).

The application of alanine, serine, or cysteine induced inward currents in a concentration-dependent manner. The responses to D-cysteine exhibited more rapid desensitization when the ligand concentration was high than those evoked by the other amino acids, and the current decreased rapidly to the baseline level (Figure 3). The concentration−response relationships of $\epsilon1\zeta1$ receptors were obtained for the peak currents evoked by coapplications of L- or D-alanine, -cysteine, or -serine and 10 μM L-glutamate (Figures 2 and 3). The EC_{50} and Hill coefficients estimated from the concentration−response relationship curves are listed in Table 2. The order of potency of the amino acids at the NMDA receptor containing the $\epsilon1\zeta1$ subunit combination was as follows: D-serine > D-alanine > D-cysteine > L-serine > L-alanine. The EC_{50} and Hill coefficients for L-cysteine were not determined because the currents evoked by L-cysteine at concentrations up to 1000 μM did not reach a plateau, indicating that receptor saturation had not been achieved. The Hill coefficients of the D-forms of these three amino acids were 1.20−1.80. The Hill coefficient of D-alanine was lower than those of D-serine and D-cysteine, suggesting that the cooperativity of D-alanine was lower than that of D-serine or D-cysteine. The Hill coefficient of D-cysteine was slightly higher than that of D-alanine or D-serine, which may indicate that cysteine interacts with the redox site of the NMDA receptor because the thiol group of the cysteine side chain has reduction−oxidization potential and reduction of the redox site of this receptor potentiates activation of the receptor (*25*).

L- and D-alanine, -cysteine, and -serine applied alone induced virtually no ionic currents (Table 1), suggesting that they activate the NMDA receptor only via the glycine-binding site.

The responses of the 38 amino acids that were tested were plotted against their molecular volumes (Figure 1). The activity changed drastically as the volume changed from that of cysteine to that of aspartate. Cysteine has a slightly smaller

Table 1: Activities of Various Amino Acids

	activity (%)[a]	*n*		activity (%)[a]	*n*
D-Ala (10 μM)	79.5 ± 11.4	5	D-Lys	1.39 ± 0.749	3
D-Ala (30 μM)	95.0 ± 12.6	4	L-Lys	0.699 ± 0.445	3
L-Ala (10 μM)	31.0 ± 23.9	4	D-Met	0.803 ± 0.221	3
L-Ala (30 μM)	57.2 ± 37.0	3	L-Met	1.37 ± 0.38	3
D-Arg	2.54 ± 4.03	3	D-Phe	1.75 ± 1.24	3
L-Arg	2.71 ± 3.32	3	L-Phe	1.52 ± 0.662	3
D-Asn	0.451 ± 0.300	3	D-Pro	3.96 ± 1.81	3
L-Asn	0.544 ± 0.260	3	L-Pro	0.871 ± 0.48	3
D-Asp	2.51 ± 1.72	3	D-Ser (10 μM)	106 ± 27.4	3
L-Asp	1.38 ± 1.34	4	D-Ser (30 μM)	92.4 ± 2.31	2
D-Cys (10 μM)	16.3 ± 18.0	6	L-Ser (10 μM)	23.3 ± 21.6	3
D-Cys (30 μM)	54.8 ± 12.9	2	L-Ser (30 μM)	49.6 ± 18.9	2
L-Cys (10 μM)	1.03 ± 1.62	5	D-Thr	0.989 ± 1.08	4
L-Cys (30 μM)	19.3	1	L-Thr	1.01 ± 0.967	4
D-Gln	0.837 ± 1.45	3	D-Trp	0.328 ± 0.333	3
L-Gln	1.19 ± 2.06	3	L-Trp	2.70 ± 0.618	3
D-Glu	4.28 ± 4.17	3	D-Tyr	1.61 ± 0.831	3
L-Glu	2.38 ± 2.97	3	L-Tyr	1.12 ± 0.408	3
D-His	4.19 ± 1.78	3	D-Val	0.891 ± 0.804	3
L-His	1.66 ± 1.51	3	L-Val	0.966 ± 1.23	3
D-Ile	1.38 ± 2.40	3	D-Ala (-L-Glu)[b]	2.72 ± 2.69	3
L-Ile	1.01 ± 1.18	3	D-Cys (-L-Glu)[b]	1.49 ± 2.27	3
D-Leu	0.803 ± 0.456	3	D-Ser (-L-Glu)[b]	2.30 ± 0.84	2
L-Leu	1.24 ± 1.09	3			

[a] Each value was the normalized current with the standard current evoked by 10 μM glycine and 10 μM L-glutamate. Activities were expressed as the mean ± standard deviation. [b] Data for D-alanine, -cysteine, and -serine with "-L-Glu" are the ratio of their current at a concentration of 1000 μM without L-glutamate.

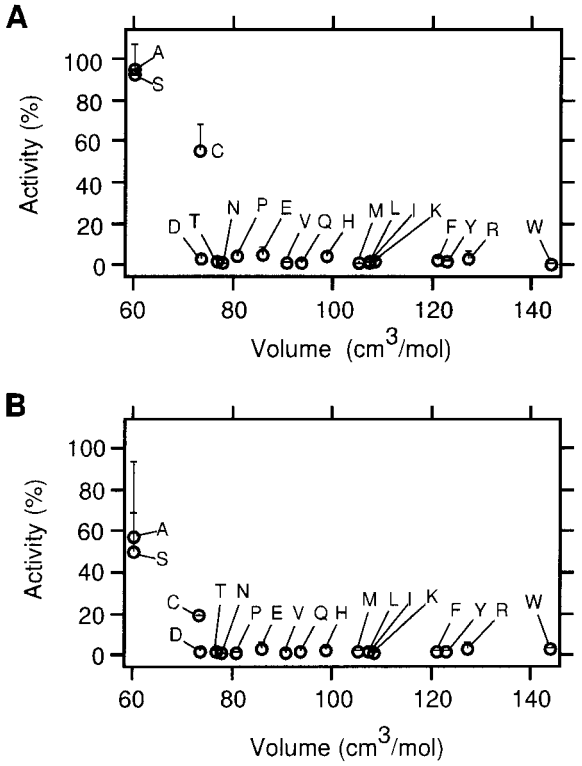

FIGURE 1: Relationships between activation of NMDA receptors consisting of ϵ1 and ζ1 subunits and the volumes of D-form (A) and L-form (B) amino acids. All the amino acids are designated by one-letter abbreviations. The activity is expressed as the ratio (as a percentage) of the current evoked by each amino acid (30 μM) and 10 μM L-glutamate to the standard current evoked by 10 μM glycine and 10 μM L-glutamate. Each error bar shows the standard deviation value. The volume data are from Zamyatnin (*32*).

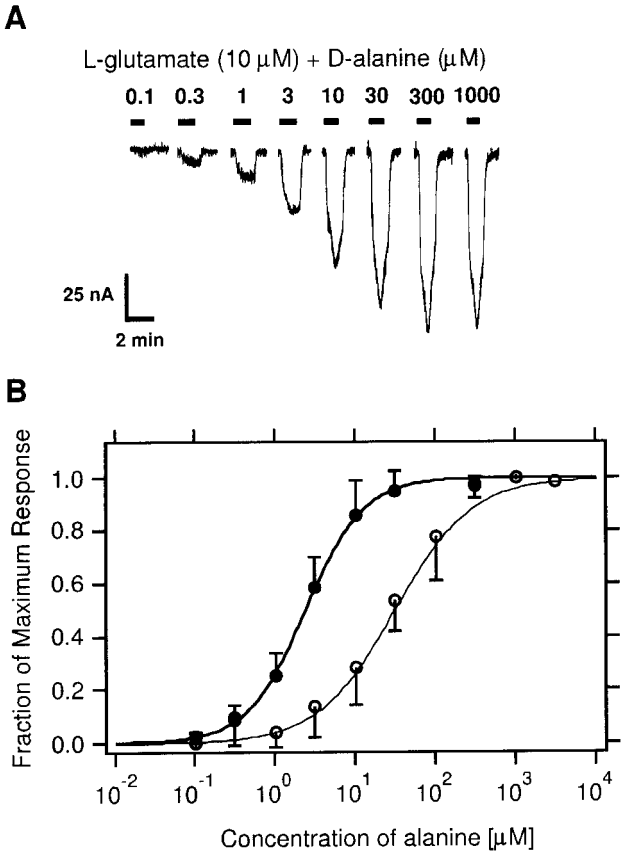

FIGURE 2: Responses to alanine of oocytes expressing ϵ1ζ1 subunits. (A) Membrane currents of oocytes clamped at -60 mV. For all the recordings, the amino acids were superfused until the current reached the maximum. (B) Concentration−response curves for D- and L-alanine in the presence of 10 μM L-glutamate. The black and white circles represent the values for D- and L-alanine, respectively. For each measurement series, the maximum current evoked was taken to be 1.0 and the current evoked by each concentration of amino acid was expressed as a fraction of this value. Each point represents the mean value obtained from three to five oocytes, and the error bar represents the standard deviation value. Curve fitting of the Hill plots for D- and L-alanine was carried out, and the EC_{50} values and Hill coefficients were determined by fitting the function $I_0 = 1/[1 + (EC_{50}/c)^n]$ to the data, where I_0 is the current normalized according to the maximum current, EC_{50} is the concentration evoking half of the maximum response, c is the concentration of agonist, and n is the Hill coefficient.

side chain than aspartate. Thus, it is suggested that the size of the amino acid is an important factor for receptor activation, and that the critical volume lies between that of cysteine and that of aspartate.

Next, to identify the factor(s) responsible for the differences in activities among these amino acids, we carried out computer simulation to investigate in detail the structural features of the amino acid molecules involved in NMDA receptor activation.

Computer Simulation of Amino Acid Molecules. The electron charge distributions in the amino acid ligand molecules were calculated by computer simulation. First, the most stable structure of each amino acid in water was calculated. Then, using their stable structures as the initial structures, the more stable structure was obtained under conditions in which all the bond lengths and bond angles were optimized. In the third step, two water molecules were placed near the amino and carboxyl groups of the ligand to simulate the formation of hydrogen bonds by the ligand with the binding site moiety. This model was based on that of Laube et al. (*22*), which shows that two amino acids at the glycine binding site of the receptor, glutamine 387 and threonine 500, interact with the amino and carboxyl groups of the ligand molecule, respectively.

Using this model, the charge distributions of alanine, β-fluoroalanine, cysteine, glycine, and serine were calculated using the WinMOPAC program. Only the charge distributions of the L-forms of the molecules were calculated, because optical isomerization does not affect the charge distribution. High positive or negative charges were located within atoms of the carboxyl and amino groups, whereas the charges carried by the side chains were low. In alanine, for example, the nitrogen atom of the amino group and the carbon and oxygen atoms within the carboxyl group carried 0.66, 0.44, -0.47, and -0.59 unit of elementary electric charge, respectively. In contrast, the charge at the β-carbon atom of the methyl group of the side chain was -0.11 unit.

The charge distributions of five amino acids were calculated, and the charge levels of the α-carbon and other atoms within the carboxyl and amino groups did not differ markedly (Figure 4B). The charges at the β-positions of their side chains, however, varied considerably. Although the absolute values at this position were not as high as those at the atoms of the carboxyl and amino groups, the signs of the values varied, e.g., -0.17 for cysteine and 0.090 for serine (Figure 5). In contrast to the charges at the β-position of the side chain, the charges of the hydrogen H9 connected to the α-carbon, which occupies the equivalent position relative to

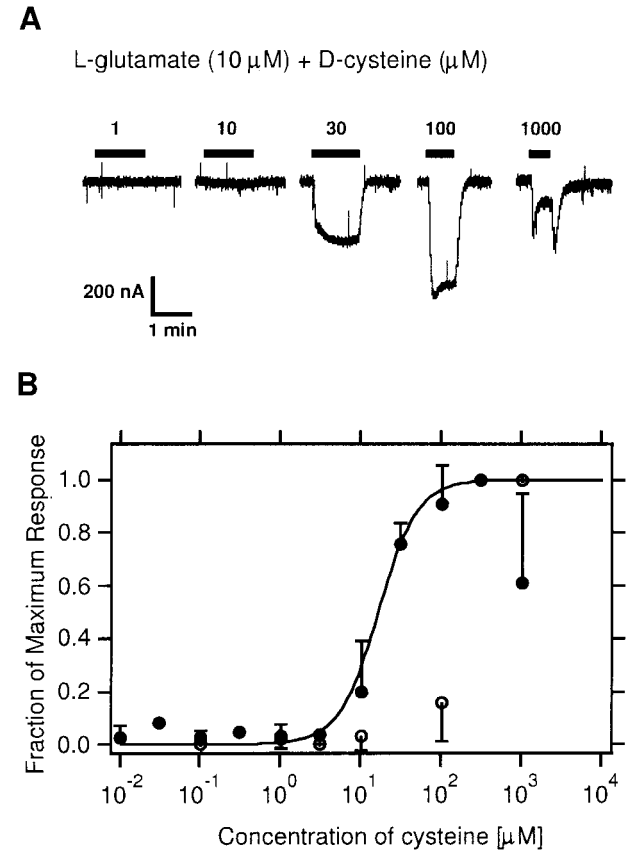

FIGURE 3: Responses to cysteine of oocytes expressing $\epsilon 1\zeta 1$ subunits. (A) Membrane currents of oocytes clamped at −60 mV. For all the recordings, the amino acids were superfused until the current reached the maximum. (B) Concentration−response curves for D- and L-cysteine in the presence of 10 μM L-glutamate. The black and white circles represent the values for D- and L-cysteine, respectively. The error bar represents the standard deviation value. Curve fitting was carried out as described above for alanine in the legend of Figure 2.

Table 2: EC_{50} Values and Hill Coefficients of D- and L-Alanine and -Serine and D-Cysteine[a]

	EC_{50} (μM)	Hill coefficient
D-Ala	2.34 ± 0.750	1.20 ± 0.358
L-Ala	29.7 ± 8.83	0.911 ± 0.192
D-Ser	0.341 ± 0.136	1.68 ± 0.550
L-Ser	69.4 ± 18.0	0.965 ± 0.167
D-Cys	16.1 ± 4.01	1.80 ± 0.502

[a] All parameters were designated as the mean ± standard deviation. They were determined by curve fitting in a Hill plot as described in the legend of Figure 2.

the α-carbon as the side chain atom to the β-position, varied little, as observed with the carboxyl and amino group atoms. Figure 6 shows the relationships between the EC_{50} values and charges at the β-position. The semilog plot for the D-form amino acids shows a strong tendency in which the higher the positive charge at the β-position, the higher the affinity of the amino acid for the glycine-binding site of the NMDA receptor. On the other hand, the curve for the L-forms is separate from that of the D-forms.

DISCUSSION

Responses to Amino Acids. In this study, the D-isomers of alanine and serine were found to be 10−100 times more

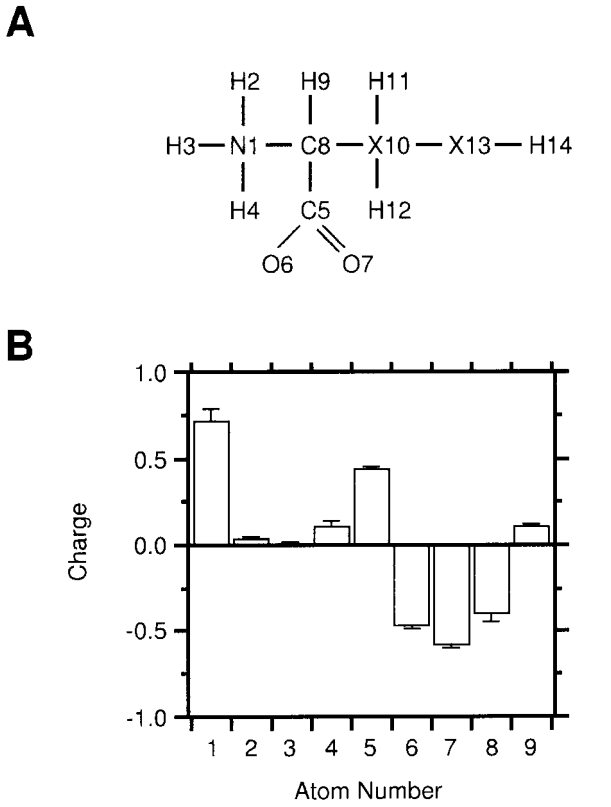

FIGURE 4: Charge distributions within common structural moieties. (A) For the numbering of the atoms within an amino acid molecule, X indicates an arbitrary atom; e.g., H10 denotes hydrogen in glycine and carbon in the other amino acids. (B) Charge distributions within the common moieties of glycine, alanine, β-fluoroalanine, cysteine, and serine. The atoms numbered 1−9 are those of the amino and carboxyl groups, the α-carbon atom, and the hydrogen atom connected to it. Each bar represents the mean value at a position among these amino acids, and each error bar represents the standard deviation.

potent potentiators of the L-glutamate response of NMDA receptors than their L-isomers. Keith et al. (*8*) showed that amino acids inhibited strychnine-insensitive glycine binding to rat cortical membrane fragments with the following rank order of potency: glycine > D-serine ≫ L-serine > β-alanine. The electrophysiological study of McBain et al. (*7*) showed that the order of relative potency of the active analogues for activation via the glycine-binding site of the NMDA receptor was as follows: glycine > D-serine > D-alanine > β-fluoro-D-alanine > L-serine > L-alanine. Our data confirm that glycine, alanine, and serine are positive modulators of NMDA receptor channels. We also found that cysteine, at a sufficiently high concentration, activated the NMDA receptor and evoked a current that was comparable to that evoked by glycine.

Structural Properties Shared by Active Amino Acids. The activating effects on the $\epsilon 1\zeta 1$ NMDA receptor of 38 amino acids in the presence of 10 μM L-glutamate were measured. Of these amino acids, only alanine, cysteine, and serine activated this receptor. The receptors $\epsilon 2\zeta 1$ and $\epsilon 3\zeta 1$ also exhibited similar properties. This finding was not surprising, since the $\epsilon 1-\epsilon 3$ subunits are highly homologous.

When these three amino acids were applied together with L-glutamate, both their D- and L-forms evoked currents comparable to that evoked by glycine, but the EC_{50} value of each D-form was lower than that of the L-form; i.e., the

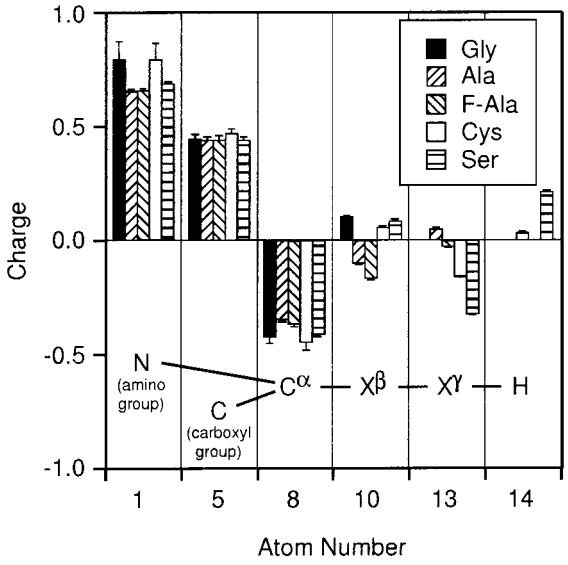

FIGURE 5: Charge distributions within amino acid molecules. The atoms carrying charges that are shown are the nitrogen of the amino group, the carbon of the carboxyl group, the α-carbon, and atoms of the side chains. The inset diagram shows the primitive structure of the amino acids. Each number indicates the position of an atom within the amino acid, and the numbering scheme is the same as that described in the legend of Figure 4A. Each bar represents the mean value at a position among these amino acids, and each error bar represents the standard deviation.

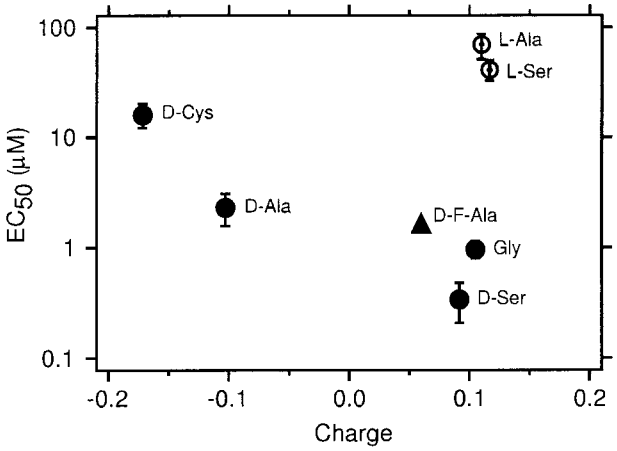

FIGURE 6: Relationships between the EC_{50} values and charges at the β-positions of ligands that are active at $\epsilon 1\zeta 1$ receptors. The EC_{50} values were plotted against the charges at the β-position, which computer simulation showed corresponds to position 10 in Figure 4A. The EC_{50} value of β-fluoroalanine that is shown was determined by McBain et al. (*7*). The black and white circles denote the data for the D- and L-forms, respectively. Each error bar represents the standard deviation.

affinities of the D-form amino acids for the NMDA receptor were higher than those of the L-forms.

A structural property shared by these three amino acids and glycine is the small size of their side chains. As described by McBain et al. (*7*), to be active at the NMDA receptor, a ligand should have carboxyl and amino groups connected to the same carbon atom. In addition to this requirement, our data suggest that the side chains of the amino acids should be small, up to three atoms without branching, for NMDA receptor activation. The numbers of atoms aligned in the side chains of glycine, alanine, cysteine, and serine are one, two, three, and three, respectively. Aspartate, which has a four-atom side chain (including the hydrogen atom at the ω-terminus), evoked a tiny current. Therefore, up to three atoms can be aligned in the side chain for activation of the NMDA receptor.

However, D-2-aminobutyric acid, the side chain of which is an ethyl group and is three atoms long, did not activate the NMDA receptor (*7*). Although the side chain of aspartate is three atoms long, it did not evoke a large current when the hydrogen at its ω-terminus was released. These findings suggest that an additional condition for activation is required. D-2-Aminobutyric acid, aspartate, and serine differ in their ω-terminal structures, i.e., methyl, carboxyl, and hydroxyl groups, respectively, indicating that the ω-terminal structure also influences NMDA receptor activation. There are one, two, and three atoms at the ω-terminus of the side chains of serine, aspartate, and D-2-aminobutyric acid, respectively. Therefore, another factor involved in receptor activation is the presence of one atom at the ω-terminus of the side chain.

Another structural feature common to the three amino acids that activated the NMDA receptor is the fact that their side chains are not branched. Part of the side chain of threonine is same as that of serine; i.e., it is three atoms long and has a hydroxyl group at its ω-terminus. However, threonine did not activate the NMDA receptor. The side chains of serine and threonine differ structurally in that the methyl group of the latter is branched, which suggests that only amino acids with unbranched side chains three atoms long with a "thin" ω-terminus can activate the NMDA receptor.

Thus, as described above, there are three essential structural features of the side chain: (1) it is up to three atoms long, (2) there is one atom at its ω-terminus when the side chain is three atoms long, and (3) it is not branched. These features explain the relationship between activity and volume shown in Figure 1, and confirm that the critical volume for activation lies between those of cysteine and aspartate.

Relationship between Receptor Sensitivities and Partial Charges of Ligand Molecules. If the essential structural features for NMDA receptor activation are those suggested above, then what is responsible for the different sensitivities of the receptor to different amino acids? The basic backbone layout of an active amino acid is characterized by (1) its carboxyl and amino groups being connected to the same carbon atom, (2) its side chain being short and unbranched, and (3) one atom being located at the ω-terminus. Therefore, the different sensitivities may depend on the internal structures of the side chains. We considered that the charge distribution within a molecule could be a structural feature that influenced receptor sensitivity, and therefore, we calculated the electron charge distributions of the amino acids using the WinMOPAC software package, for which the calculation engine is MOPAC97.

The results revealed subtle differences in charge distribution within the atoms of the amino and carboxyl groups, the α-carbon, and the hydrogen atom connected to it (atoms 1−9 shown in Figure 4A) among glycine, alanine, β-fluoroalanine, serine, and cysteine (Figure 4B). Therefore, the major charge distribution differences could not be attributed to the different charges carried by these moieties.

The atom at the β-position in the side chain is the only one common to the side chains of glycine, alanine, cysteine, and serine. Therefore, the relationship between the calculated

electron charge at the β-position and the EC_{50} of each ligand molecule was examined. The charges exhibited a linear relationship to the logarithm of the EC_{50} with a negative slope; i.e., the sensitivity of the receptor to amino acid ligands increased as the positive charge at the β-position atom increased. If it is assumed that all the Hill coefficients of these active amino acids are identical, the logarithm of the EC_{50} value is the Gibbs free energy multiplied by a constant (*32*). Therefore, this charge−EC_{50} relationship suggests that the receptor sensitivity is based on the electronic interaction between the charge of the atom at the β-position of the ligand side chain and that of the glycine-binding site of the receptor.

This result is consistent with that of Kuryatov et al. (*21*), whose model of the glycine-binding site is based on the three-dimensional structure of lysine/arginine/ornithine binding protein (LAOBP) (*26*). They recorded currents generated by mutated receptors and showed that some charged residues were located at the glycine-binding site. They used the rat NR1 subunit, which is highly homologous with the mouse ζ1 subunit, and therefore, their results should be applicable to ours with the mouse ζ1 subunit.

The charged residues at the glycine-binding site may interact with the charged β-atoms of active ligand molecules. Even though the charge at this position is much smaller than those carried by the amino and carboxyl groups of the ligands, the environment within the glycine-binding site, where, according to Kuryatov's model, the charged residues are embedded in a hydrophobic environment, may enhance the interaction between the charges of the β-atom and the glycine-binding site. In such an environment, the dielectric constant is lower than that in a hydrophilic environment and, therefore, the electron potential can extend further and even the small charge carried by a ligand molecule can interact strongly with the charged residues surrounded by hydrophobic residues (*27*, *28*). The hydrophobic environment may enhance the interaction between the ligand side chain carrying a small charge and the charged residues in the binding site. This mechanism would explain the effect of a weak charge on the ligand binding affinity of the NMDA receptor.

Here we found a positive relationship between the ligand affinity and the charge at the β-position of the ligand. However, the curvature was not so smooth. Therefore, there would be some unknown factors that perturb the interaction between the ligand and the receptor binding site.

Other Structural Features of the Glycine-Binding Site. If it is considered that not only the D-form of the active amino acids but also their L-forms activate the receptor, even though a high ligand concentration is required, other mechanism(s) seem to be involved in receptor activation. Under the assumption that the basic alignment of a ligand with the glycine-binding site is determined by the orientation of the carboxyl and amino groups of the amino acid ligand relative to the binding site, the β-positions of D-form amino acid side chains will always correspond to the hydrogen atoms of the L-forms. If the same mechanism determines the receptor's sensitivity to L- and D-form amino acids, then the curves of the EC_{50} values against the charges at the hydrogen atom at the β-position should have been superimposed, which they were not. This suggests that other mechanisms determine the receptor sensitivity in addition to the spatial layout of the amino and carboxyl groups and the atom at the β-position of the ligand side chain.

The most likely factor is the small size of the glycine-binding site. As described above, the activities of D-form amino acids are determined by the sizes of their side chains, which suggests that the glycine-binding site consists of a small pocket (*22*). The three-dimensional structure of LAOBP, on which the three-dimensional structural model of the glycine-binding site was based, has a small ligand-binding site, and bacterial leucine/isoleucine/valine binding protein (LIVBP), a protein homologous with LAOBP, also has a small ligand-binding pocket in the three-dimensional structure of its LIVBP−ligand complex (*29*). Such a small space at the binding site could restrict the ligand configuration inside the pocket, thereby perturbing the affinity of the L-form amino acid. The affinity of the ligand should be basically determined by the interaction between the charges of the hydrogen at the β-position and of the receptor, if there is no spatial restriction when the ligand binds to the binding site.

Another factor that would account for the low activation potencies of L-form amino acids is hydrophobic interaction. As described above, as there are several aromatic residues around the glycine-binding site, the side chain of a ligand and the inside wall of the glycine-binding site may interact in a hydrophobic manner. This may account for the separation of the curves of the charge against the negative logarithm of the EC_{50} for the L- and D-amino acids.

The structures of many antagonists of the glycine binding site suggest that their binding is configured in the same manner as that of the ligand amino acids. Most antagonists have moieties similar to the amino and carboxyl groups. This structural feature would allow them to bind to the glycine site. However, their other structural moieties are quite different from those of the agonists. Primarily, they are large. Such large moieties could not fully enter the binding site, and even if this were possible, they would not change the receptor conformation required for activation. Therefore, two functional parts may exist within the glycine binding site: one for ligand binding and the other for activating the receptor.

Our study has elucidated several structural features of the amino acid ligands and of the glycine-binding site of the NMDA receptor. To investigate this receptor further, it will be necessary to obtain more detailed structural information, such as its atomic coordinates determined by X-ray crystallography and/or nuclear magnetic resonance (NMR) methods. Structural data about even a fragment of the glycine-binding site would provide valuable information about the mechanism(s) responsible for amino acid ligand binding to the NMDA receptor (*30*).

ACKNOWLEDGMENT

We are grateful to Drs. Mitsuhiko Matsumoto and Shingo Kimura for performing preliminary experiments and to Dr. Takeshi Kikuchi for helpful discussion about computer simulation.

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BI990246K