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European Journal of Pharmacology 312 (1996) 357–365

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Binding of the radiolabeled glycine site antagonist [^{3}H]MDL 105,519 to homomeric NMDA-NR1a receptors

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Received 11 April 1996; revised 14 June 1996; accepted 18 June 1996

Abstract

We have characterized the binding of [^{3}H]MDL 105,519 ((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid), a NMDA receptor glycine recognition site antagonist, to homomeric NMDA subunit 1a (NR1a) receptors. Chinese hamster ovary cells (CHO-K1) were transfected with the rat NR1a gene and cell lines stably expressing the receptor were isolated from amongst clones resistant to the neomycin analog G418. Saturation analysis indicated that the radioligand bound to the homomeric receptor with a similar high affinity (K_d = 1.8 nM) to that reported for the native receptor. The binding capacity (B_{max}) was 370 fmol/mg protein reflecting approximately 110 000 receptors per cell. The radioligand interacted with a single class of binding sites as indicated by linear Scatchard transformation of the saturation data and a unitary Hill slope in competition experiments. Thus, the MDL 105,519 recognition site is present on the NR1a subunit and has similar radioligand binding properties to the native brain-derived receptor. However, pharmacologic characterization of [^{3}H]MDL 105,519 binding indicated that agonists were weaker competitors at the homomeric receptor relative to the native receptors. In contrast, representatives of three distinct chemical classes of glycine site antagonists exhibited similar potencies at both types of binding sites.

Keywords: NMDA receptor; [^{3}H]MDL 105,519; Glycine; (Binding)

1. Introduction

NMDA receptors are ligand-gated cation channels composed of at least two different polypeptide subunits suggested to be arranged as pentameric complexes (Nakanishi, 1992; Hollmann and Heinemann, 1994; Seeburg et al., 1995). These receptors are highly permeable to Ca^{2+}, are voltage dependently regulated by Mg^{2+}, and play key roles in neuronal signal processing. The binding sites for the co-agonists glutamate and glycine offer therapeutic opportunities to develop NMDA receptor site-specific antagonists which are proving to be neuroprotective in animal stroke models (Hasegawa et al., 1994; Warner et al., 1995) and in the clinic (Lipton and Rosenberg, 1994; Muir and Lees, 1995).

The NR1 subunit, which contains the co-agonist binding sites for glutamate and glycine (Laurie and Seeburg, 1994), is present in all NMDA receptors and interacts with one or several NR2 subunits (A,B,C,D; each encoded by a different gene) to give heteromeric complexes with distinct electrophysiological or pharmacological characteristics (Ishii et al., 1993; Laurie and Seeburg, 1994; Priestley et al., 1995). Physiological heterogeneity is further enhanced by the NR1 gene being differentially spliced yielding 8 different polypeptides reflecting all possible combinations of 3 different, independently occurring splice events. These splice variants display different agonist and antagonist potencies, as well as differences in the size of currents, sensitivities to zinc ions and modulation by protein kinases (Sugihara et al., 1992; Zukin and Bennett, 1995).

The NR1a gene encodes the predominant isoform in the adult rat brain and was first cloned by Moriyoshi et al. (1991) via expression cloning. Translation of the NR1a mRNA in *Xenopus* oocytes reproduces the pharmacological characteristics of the native receptor, though with a much-diminished ion conductance. Using this expression system, NMDA electrophysiological characteristics were found to be dramatically enhanced by the co-expression of any of the NR2 mRNAs. However, rat homomeric NR1a receptors expressed in mammalian cells indicate a lack of functional NMDA receptors when studied with whole-cell patch clamp analysis (Boeckman and Aizenman, 1994). Grimwood et al. (1995a) have transiently expressed the human NR1a and NR1e genes in human embryonic kidney

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PII S0014-2999(96)00477-3

cells (HEK293) and confirmed that the homomeric receptor was non-functional as evidenced by electrophysiology and lack of cell death in glutamate-containing culture media. These investigators employed radioligand binding analysis with [^{3}H]L-689,650, ((±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline), another NMDA receptor associated glycine recognition site antagonist, to demonstrate the presence of a high-affinity glycine antagonist binding site in human homomeric NR1a and NR1e receptors.

In this report, we characterize the binding properties of a chemically distinct radiolabeled NMDA receptor glycine recognition site antagonist [^{3}H]MDL 105,519 ((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid, Baron et al., 1996) to the homomeric rat NMDA-NR1a receptor stably expressed in Chinese hamster ovarian cells (CHO-K1). In addition, we have used this radioligand to evaluate the pharmacology of the homomeric NMDA-NR1a receptor as defined by competition binding versus several unlabeled glycine site agonists and antagonists.

2. Materials and methods

2.1. Subcloning the rat NR1a gene

The rat NR1a gene (generously provided by Dr S. Nakanishi, Kyoto University, clone pN60) was excised from pBluescript with the two blunt end cutting restriction enzymes Nru I and EcoR V. This fragment contains the entire coding region of the NR1a gene flanked by 53 bp of 5′ untranslated nucleotides and 257 bp of 3′ untranslated nucleotides of the NR1a gene. We presumed that these remaining untranslated nucleotides would not significantly affect expression of NR1a coding sequences in the expression vector. This fragment was then ligated into the EcoR V site of the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). The correct orientation of the insert was ascertained by restriction mapping using EcoR I (1080 bp + 7493 bp fragments) and BamH I/EcoR V (3170 bp + 5403 bp fragments). The subcloning protocols used were standard recombinant DNA techniques as outlined in Maniatis et al. (1982).

2.2. Cell culture and transfection

CHO-K1 cells (ATCC CCL 61) were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12(Ham) 1:1 (GIBCO, D-MEM/F-12 medium) supplemented with 10% Fetal Bovine Serum, 5 mM Hepes (*N*-[12-Hydroxyethyl] piperazine-*N*-[2-ethanesulfonic acid]), 1 mM L-glutamine, 50 U/ml penicillin G sodium, 50 μg/ml streptomycin sulfate and 0.125 μg/ml amphotericin B, pH 7.1. On day one, cells were collected from two 150 cm^2 confluent flasks (~ 3×10^6 cells per flask) with 10 ml Versene (GIBCO) to detach the cells which were then collected by centrifugation at $1000 \times g$ for 5 min. The pelleted cells were resuspended into 6 ml Opti-Mem I Media (GIBCO) and placed on ice. Electroporation was performed using a Gene Pulser Transfection Apparatus (BioRad Labs, CA, USA), 0.8 ml cells and 20 μg pcDNA3 or pcDNA3-NR1a (circular or linearized with Pvu I) in cuvettes with 0.4 cm electrode gap, at settings of 0.45 V and 500 Ω capacitance. Electroporated cells were incubated on ice for 15 min, diluted into 25 ml complete D-MEM/F-12 medium, and allowed to grow for 48 h. On day 3, the cells were detached with 5 ml Versene and 0.8 ml cells added to each of six 100 mm^2 dishes with 15 ml D-MEM/F-12 complete medium containing 200 μg/ml G418 (pH 7.1). The concentration of G418 was increased on day 6 (800 μg/ml) and day 9 (1200 μg/ml). On day 12 all cells which had been electroporated with pcDNA3 were dead. Individual colonies were picked from the pcDNA3-NR1a treated plates with sterile plastic pipette tips and transfered to 6 well plates containing 2 ml D-MEM/F-12 complete media per well. G418 was reduced at this time to 600 μg/ml. Confluent wells were then passed to 100 mm^2 dishes and grown to confluency. Cells were collected with 5 ml Versene, centrifuged, and taken up into 1 ml 50 mM Tris-acetate, pH 7.4 (Buffer A), polytroned (Kinematica PT1200, setting 5, 10 s) and stored at −80°C until [^{3}H]MDL 105,519 binding was performed.

Once the colony with the highest specific binding was identified, we routinely grew the NR1a transfected cell line (denoted C-1) in 225 cm^2 flasks in the presence of 200 μg/ml G418, and split the cells 1 to 20 upon reaching confluency. Throughout these procedures, unattached cells were routinely removed prior to treatment with Versene by washing the well or flask once or several times with 1 to 10 ml Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (GIBCO).

2.3. Membrane isolation

Cells were detached from confluent 225 cm^2 flasks with 10 ml Versene, collected at $1000 \times g$ for 5 min and the cell pellet resuspended in ice cold phosphate buffered saline, and homogenized via a polytron (Brinkman PT10/35, setting 6, 10 s). The homogenates were centrifuged ($40\,000 \times g$ for 20 min, 4°C), the resulting pellet was suspended using the polytron in 40 ml ice cold buffer A, and recentrifuged. The final membrane pellet was suspended into 15 ml ice cold buffer A and stored as 3 ml aliquots at −80°C. Protein determinations were performed using the dye method of Bradford (1976).

2.4. Western blots

Membrane fractions prepared in the presence of proteinase inhibitors (1 μg/ml aprotinin, 0.5 μg/ml leuptin, 0.5 mg/ml EDTA-Na2: Metalloproteinases inhibitor and 0.7 μg/ml pepstatin; Boehringer Mannheim, Indianapolis, IN, USA) from control rat brain cortices (used as positive

controls for NR1 expression) and CHO-K1 cells were subjected to electrophoresis on 16% sodium docecyl sulfate (SDS)-polyacrylamide gel under reducing conditions. Proteins were electroblotted for 4 h at 46 V (186 mA) in 25 mM Tris base-192 mM glycine-10% methanol buffer onto a Westran PDVF protein transfer membrane (Schleicher & Schuell, Keene, NH, USA). The protocol used for immuno-hybridization and detection was that provided by the supplier of the Westran membrane. Blotted membrane was rinsed with water, and incubated for 12 h at 4°C in the pre-hybridization solution (5% bovine serum albumin (BSA), 0.05% Tween-20 in phosphate buffered saline (PBS), following which it was incubated for 5 h at room temperature in the hybridization solution (2.5% BSA, 0.05% Tween-20, and 0.5 μg/ml of rabbit anti-NMDAR1 polyclonal antisera AB1516 (Chemicon International, Temecula, CA, USA) in PBS. This antisera was made to the LQNQKDTVLPRRAIEREEGQLQLC-SRHRES synthetic peptide, corresponding to the C-terminus of the rat NMDA receptor NR1a subunit. Following hybridization, the filter was washed three times (5–10 min per wash) with 75 ml of PBS containing 0.05% Tween-20 and incubated for 2 h at room temperature in the second hybridization solution (2.5% BSA, 0.05% Tween-20 and 1 μg/ml of goat anti-rabbit IgG-alkaline phosphatase secondary antibody AP132A (Chemicon) in PBS). The filter was subsequently washed three times (5–10 min per wash) with 75 ml of 50 mM Tris-base pH 7.4 in 0.2 M NaCl containing 0.05% Tween-20, 0.1 mM $MgCl_2$ and 0.4 μM $ZnCl_2$. The washed membrane was then incubated with 10 ml of ready mix stable NBT/BCIP solution (Life Technologies, Gaithesburg, MD, USA) for detection of the antigen-antibody complex.

2.5. Binding assays

[^{3}H]MDL 105,519 binding was performed in borosilicate glass tubes. The assay was performed in buffer A (50 mM Tris-acetate, pH 7.4), with a final volume of 0.5 ml. Stored frozen membranes were thawed, polytroned briefly and used directly. Following incubation for 30 min at room temperature bound ligand was then collected by filtration onto GF/B glass fiber filters (Whatman) using a Brandel Cell Harvester. The collected membranes were washed three times with 5 ml ice cold buffer A, placed into Beckman mini-vials, and radioactivity was quantitated in 4 ml Readiprotein (Beckman) by liquid scintillation spectroscopy. The radioligand concentration was 0.1–40 nM for saturation studies and 4 nM in competition binding experiments. Stock solutions of all compounds tested were dissolved in dimethylsulfoxide (DMSO) or 10% DMSO/H_2O such that the final concentration of DMSO in the binding assay was 0.1%, a concentration shown in control tubes not to interfere with [^{3}H]MDL 105,519 binding to the membranes. Non-specific binding was defined as that remaining in the presence of 1 mM unlabeled glycine.

Procedures for [^{3}H]glycine binding and the rat brain membrane preparation for native NMDA receptor comparisons have been previously described in Baron et al. (1991). [^{3}H]Glutamate binding was performed as a centrifugation assay similar to the [^{3}H]glycine binding assay with the following conditions: 50 mM Tris-HCl buffer, pH 7.4, 20 nM [^{3}H]glutamate (Dupont New England Nuclear, Boston, MA, USA; specific activity of 17.8 Ci/mmol), 30 min on ice, and 1 mM L-glutamate was used to define non-specific binding.

2.6. Data analysis

All data were analyzed as per Limbird (1986) as described in Baron and Siegel (1990), using custom-written routines in Microsoft Excel spreadsheet software. Statistical analysis was performed using StatView software and employed one or two way analysis of variance (ANOVA) followed by post-hoc comparisons with Fisher's probability least significant difference test. The criteria for statistical significance was a P value less than 0.05. Unless specified otherwise, results are reported as means ± S.E.M. for 'n' replicates. Saturation data were fit as follows. Non-specific binding at concentration 'x' was fit to the line $f(x) = ax + b$ using linear regression. Total binding was then modeled using non-linear regression as the sum of non-specific and saturable components with the equation $f(x)ax + b + mx/(k + x)$. The terms m and k represent computed values for the maximal binding (B_{max}) and the equilibrium dissociation constant (K_d), respectively. Competition binding was fit using non-linear regression to the model 'inhibition' $= 100x^n/(k^n + x^n)$, where $n =$ slope.

2.7. Chemical sources

[^{3}H]MDL 105,519 (specific activity 71 Ci/mmol) was synthesized by Amersham International, Wales, UK. Unlabeled MDL 105,519 ((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1*H*-indole-2-carboxylic acid), ACEA1021 (Woodward et al., 1995; 5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione) and L-701,324 (Kulagowski et al., 1994; 7-chloro-4-hydroxy-3-(3-phenoxy)phenylquinolin-2(1*H*)one) were synthesized at Hoechst Marion Roussel. AP5 (2-amino-5-phosphonopentanoic acid), AP7 (2-amino-7-phosphonoheptanoic acid), AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid), kainic acid, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), and all other chemicals unless noted otherwise were obtained from Sigma (St. Louis, MO, USA).

3. Results

3.1. Clonal selection

Following the transfection protocol outlined above, we selected 49 colonies, 41 of which grew well enough to

isolate sufficient membranes to measure binding of [^{3}H]MDL 105,519. In each assay, we concurrently monitored ligand binding to a rat brain membrane preparation as a positive control. The best lines were expanded and maintained or cryopreserved in liquid nitrogen. All binding studies reported here are from clone C-1, the selection of which was based on the level of specific binding.

3.2. Immunoblots

The results of the immunoblot analysis (Fig. 1) clearly indicate that the membrane fractions prepared from the CHO-K1 cell clone C-1 stably transfected with pcDNA3-NR1a contains NR1 antibody reactive material in the $M_r \sim 120000$ dalton region of the gel. This band co-migrates with authentic NR1a obtained from rat brain. Also, as expected, the membrane fraction from the control CHO-K1 cells is clearly negative for NR1a gene expression. The expression level of NR1a in cell line C-1 is comparable to that with the rat brain sample.

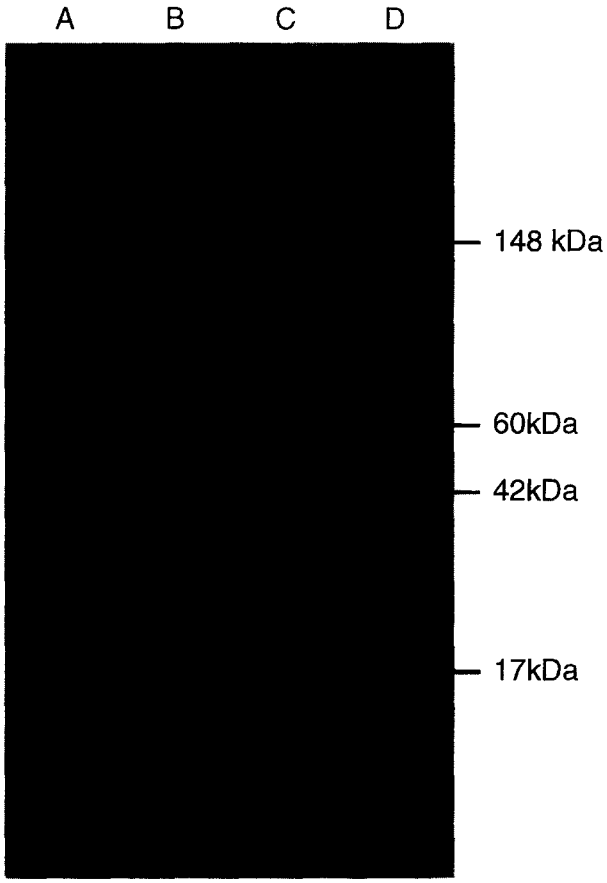

Fig. 1. Immunoblot of rat brain cortical membranes and CHO-K1 cell membranes transfected with pcDNA3-NR1a, probed with anti-NMDAR1 antibodies. Approximately 3.2 μg protein equivalent of membranes were electrophoresed on 16% SDS-Polyacrylamide gel and subjected to immuno-blot analysis as detailed in the text. The molecular weights indicated were determined by electrophoresing prestained molecular weight standards (Novex Novel Experimental Technology, San Diego, CA, USA) on the same gel. Lane A is of membranes prepared from stable CHO-K1 cell transfectant clone C-1 and lanes B and C contain membrane preparations from rat brain and untransfected CHO-K1 cells, respectively.

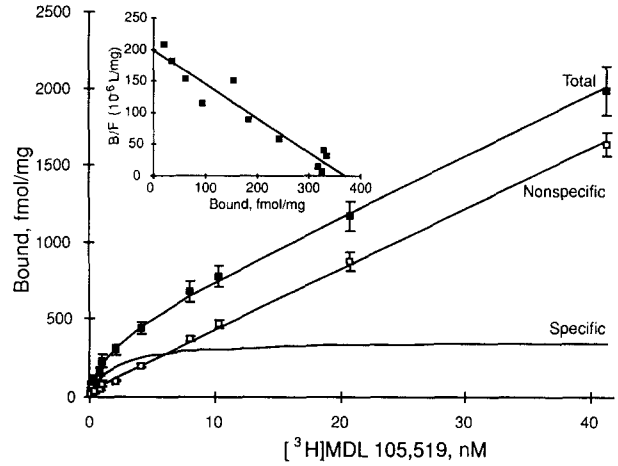

Fig. 2. Saturation isotherm and Scatchard transformation (inset) of [^{3}H]MDL 105,519 binding to membranes CHO-K1 cells stably expressing the NR1a gene. Non-specific binding was defined in the presence of 1 mM glycine. The data shown are pooled data (means ± S.E.M.) obtained in three independent experiments.

3.3. Saturation analysis

Association studies (not shown) were performed at room temperature and verified that the binding of [^{3}H]MDL 105,519 (4 nM) to the homomeric receptor reached asymptotic levels by approximately 10 min, exhibiting similar kinetics as the native rat brain NMDA receptor. Further studies utilized a 30 min incubation period as defined for native NMDA receptors (Baron et al., 1996).

Saturation analysis to CHO-NR1a membranes was performed using 0.1 to 40 nM [^{3}H]MDL 105,519 (Fig. 2). Binding was saturable with K_d of 1.84 ± 0.27 and B_{max} of 370 ± 50 fmol/mg protein ($n = 3$). The data transformed by the method of Scatchard was well approximated with a straight line indicating that [^{3}H]MDL 105,519 was interacting with a single population of binding sites. When calculated using a model allowing for a variable slope, the Hill coefficient of the saturation isotherm was 1.22 ± 0.06 ($n = 3$), a value not significantly different from unity ($P > 0.05$). Values for K_d and B_{max} in this analysis were 1.64 ± 0.26 nM and 350 ± 60 fmol/mg protein respectively. The observed receptor density in the membrane preparation corresponded to 110000 ± 12800 [^{3}H]MDL 105,519 binding sites per cell. Percent specific dpm bound was 66% total dpm bound at the K_d.

For comparison, we also measured the binding of 50 nM [^{3}H]glycine or 60 nM [^{3}H]glutamate with these NR1a membranes. Only small amounts of specific binding were observed, corresponding to approximately 7% ([^{3}H]-glycine) and 1% ([^{3}H]glutamate) of the magnitude of specific binding seen in a rat brain membrane preparation used as a positive control (see Table 1). More detailed

Table 1
Comparison of the amount of binding of [^{3}H]MDL 105,519, [^{3}H]glycine and [^{3}H]glutamate to rat homomeric NMDA-NR1a receptors and native rat brain membranes

NMDA receptor type	[^{3}H]MDL 105,519 (2 nM)	[^{3}H]Glycine (50 nM)	[^{3}H]Glutamate (60 nM)
Homomeric NR1a	370± 50 [a]	47±14 [b]	49± 14 [b]
Native rat brain	8200±412 [a]	702±53 [b]	4391±114 [b]

Data reported as fmol radioligand bound per mg protein. [a] Means±S.E.M. of 4 separate expreriments performed in quadruplicate. [b] Mean±S.E.M. for 1 experiment performed in quadruplicate.

studies to examine whether the apparent discrepancies arose from differences in site density or affinity were not performed.

3.4. Pharmacology of agonists and antagonists

A number of NMDA receptor-associated glycine recognition site agonists and antagonists were tested for their ability to compete for [^{3}H]MDL 105,519 binding to the CHO-NR1a membranes. K_i values were calculated by the Cheng-Prusoff method using the measured K_d of the radioligand and its concentration in the binding assay. As shown in Table 2, the agonists glycine and D-serine displayed a 19- and 5-fold greater K_i value (lower affinity) compared to the respective values for native NMDA receptors in rat brain membranes (see Fig. 3). In contrast, the K_i values obtained for antagonists compared well with those previously obtained for inhibiting [^{3}H]MDL 105,519 binding to rat cortex/hippocampal P_2 membranes (Baron et al., 1996). Hill slopes were in each case not significantly different from unity (one way ANOVA followed by Fisher's probability of least significant difference test with a P value set to 0.05) indicating simple mass action kinetics.

While each of the competing ligands could fully inhibit the specific binding of the radioligand, unlabeled MDL 105,519 (or closely related analogs, data not shown) exhibited maximal inhibition values of greater than 100%. As

Table 2
Pharmacological characteristics of [^{3}H]MDL105519 binding to homomeric NMDA-NR1a receptors

Compound	K_i, nM	n_{Hill}	Ratio K_i values NR1a/Native
Glycine	2610±279	0.91±0.12	18.9 [a]
D-Serine	1241±229	0.89±0.17	4.7 [a]
MDL 105,519	1.4±0.3	1.04±0.10	0.33 [a]
ACEA 1021	5.3±0.7	0.91±0.17	0.74 [a]
L-701,324	4.9±1.1	0.81±0.25	3.5 [b]

[a] Data for native receptors are from Baron et al. (1996). [b] K_i value for L-701,324 at native receptor was 1.4±0.1 nM (n = 3). Results for NR1a are means±S.E.M. of 4 independent experiments.

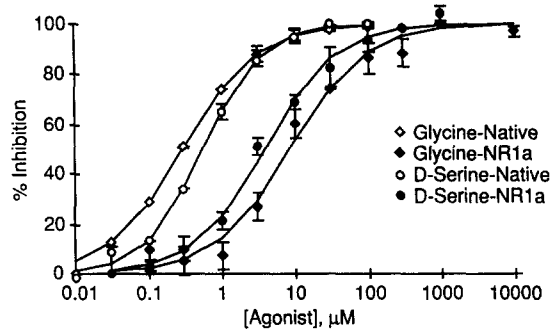

Fig. 3. Relative affinity of glycine site agonists for native versus homomeric NR1a NMDA receptors. [^{3}H]MDL 105,519 competition binding experiments are shown for the agonists glycine (diamonds) and D-serine (circles). Affinity was measured using recombinant NMDA-NR1a receptors (closed symbols, n = 4) or native receptors obtained from rat brain (open symbols, n = 3). The results shown are the means±S.E.M. of three independent experiments.

these results may indicate the existence of a saturable and possibly NMDA receptor-unrelated contribution to binding, they were addressed in more detail.

[^{3}H]MDL 105,519 binding was measured in the presence or absence (total binding) of high concentrations of unlabeled ligands using either membranes prepared from the NR1a-containing or wild type CHO-K1 cell lines. The amount of binding (fmol/mg protein, mean ± S.E.M., n = 3 experiments) observed in the presence or absence of the unlabeled ligands was analyzed by means of two way analysis of variance (unlabeled ligand × cell line) and is shown in Fig. 4. The analysis revealed a significant (P < 0.001) effect of both unlabeled ligand type and of the cell line used as well as a significant (P < 0.001) interaction

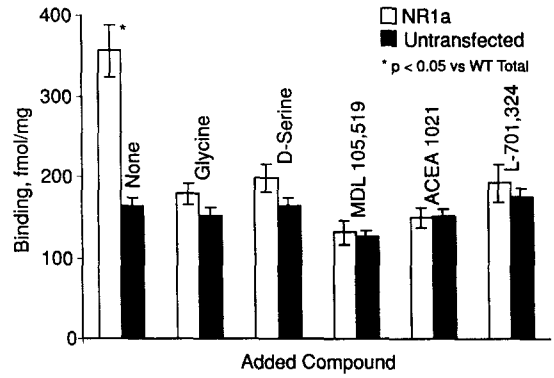

Fig. 4. Unlabeled NMDA glycine antagonists reduce [^{3}H]MDL 105,519 binding to the NR1a subunit to untransfected levels. The level of [^{3}H]MDL 105,519 binding was measured in the absence (total binding) (open bars) or presence (solid bars) of various unlabeled ligands: glycine (1 mM), D-serine (1 mM), MDL 105,519 (10 μM), ACEA 1021 (10 μM) or L-701,324 (10 μ). Cells expressing the NR1a gene had higher levels of total binding which was reduced in the presence of the unlabeled ligands to wild type control levels (i.e. wild type total binding). Results shown are the means±S.E.M. of three independent experiments. Statistical significance (two way ANOVA followed by Fisher's probability of least significant difference test) is indicated by an asterisk.

between the two variables. These results are consistent with the appearance of NMDA receptor-like pharmacological characteristics in cells stably expressing the NR1a construct. Post hoc comparisons were made using the Fisher's probability of least significant difference test and pertinent contrasts are illustrated usings asterisks ($P < 0.05$) in Fig. 4. As can be seen, the NR1a-containing membranes had higher levels of binding which were reduced to a similar extent in the presence of competing ligands. There were no significant differences between the level of binding observed in the presence of the different unlabeled ligands, all reducing binding to the level observed in the untranfected wild type CHO-K1 cell membranes. However, these considerations underscore the importance of appropriately defining specific binding, particularly in instances of low site density. As described in Methods, unlabeled 1 mM glycine was used throughout the studies reported here.

3.5. Lack of allosteric interactions

[^{3}H]MDL 105,519 binding to homomeric NR1a receptors expressed in CHO-K1 cells was not influenced by the glutamate recognition site ligands L-glutamate, NMDA, D-AP5, and D-AP7 at concentrations (1 to 1000 μM; data not shown) known to allosterically modulate binding of another NMDA receptor-associated glycine site antagonist radioligand, [^{3}H]L-689,650 (Grimwood et al., 1995a), at the NMDA receptor of rat brain membranes. The non-NMDA agonists AMPA and kainic acid (10 and 100 μM) also did not influence [^{3}H]MDL 105,519 binding to the rat NR1a homomeric receptors (data not shown), although the AMPA antagonist CNQX did have a K_i comparable to that found with native rat brain NMDA receptors (K_i = 3.0 ± 0.4 μM vs. 6.2 ± 0.2 μM respectively, $n = 3$).

4. Discussion

The expression of recombinant heteromeric NMDA receptors with defined subunit compositions (Laurie and Seeburg, 1994; Priestley et al., 1995) provide models to identify pharmacological agents which possess selectivity for different NMDA receptor subunit combinations. Selectivity has been demonstrated pharmacologically in in vivo behavioral studies among several NMDA receptor-associated glycine recognition site antagonists (Kehne et al., 1995). A better understanding of the co-agonist binding sites (Wafford et al., 1995) of the NR1 subunits will provide useful information in elucidating co-agonist allosteric interactions of both homomeric NR1 receptors and heteromeric NMDA receptors. In this study, we have stably expressed the rat NMDA-NR1a cDNA in CHO-K1 cells and established some of the fundamental characteristics of the binding of the NMDA receptor glycine site antagonist [^{3}H]MDL 105,519. These experiments establish the conditions for using this radioligand in recombinant systems as a pharmacological tool to probe the glycine recognition site and define its molecular features.

4.1. Saturation analysis

The binding of [^{3}H]MDL 105,519 to homomeric rat NR1a receptors was saturable and of high affinity. Scatchard transformation indicated the radioligand bound to a single population of receptors in the stable cell line. The measured K_d value (1.84 nM) was very similar to that previously reported for the native receptor of rat brain (K_d = 3.77 nM, Baron et al., 1996). These results indicate that the NR1a subunit expresses a recognition site for the radiolabeled antagonist which is similar to that found on native membranes.

Grimwood et al. (1995a) found a similar affinity of the NMDA receptor glycine recognition site antagonist [^{3}H]L-689,650 for human homomeric NR1a receptors transiently expressed in HEK293 cells. These receptors were expressed at a higher level (B_{max} = 3.82 pmol/mg protein) than the rat NR1a receptor expressed in CHO-K1 cells, reflecting either a difference in species or cell type used for the transfections. [^{3}H]L-689,650 binds to a single population of binding sites using homomeric NR1a receptor preparations but appears to exhibit more complex interactions with native NMDA receptors. For example, using brain membranes, [^{3}H]L-689,650 saturation binding exhibited Hill coefficients which were significantly greater than unity and followed multiexponential association and dissociation kinetics (Grimwood et al., 1993). These authors suggest that the predominant site in the native receptor population resembles the antagonist preferring state described by Monaghan et al. (1988). In contrast, unitary Hill coefficients were observed for antagonist interaction with recombinant NR1a subunits in both the present study using [^{3}H]MDL 105,519 and in that of Grimwood et al. (1995a) using [^{3}H]L-689,650 binding.

4.2. Immunoblots

Western blots of membranes isolated from the CHO-NR1a C-1 clone gave a single reactive band to the NMDA-R1 antibody at $M_r \sim 120000$ daltons which was identical in gel mobility to the single reactive band of native rat brain membrane. Chazot et al. (1992) transiently expressed the rat NMDAR1a gene in HEK293 cells and using an antibody against the C-terminal 10 amino acids detected two reactive bands at M_r = 117000 and 97000 daltons. Treatment of their membranes with *N*-glycosidase converted all the glycosylated 117000 dalton band to a non-glycosylated 97000 dalton band. Probably our failure to detect a 97000 dalton band in the CHO-K1 cells is a reflection of differences between the 2 host cell types in their efficiency or extent of post-translational processing events which lead to the mature glycosylated receptor.

4.3. Pharmacology

Using the radiolabeled antagonist [^{3}H]MDL 105,519, we have investigated some of the pharmacological properties of the rat homomeric NR1a subunit. The glycine site agonists glycine and D-serine exhibited lower affinities for the recombinant NR1a receptors than the native NMDA receptors. It is of interest that the affinity of D-serine for the homomeric receptor more closely approximated its affinity for the brain receptor whereas glycine was dramatically less potent. It is possible that the availability of an additional hydrogen binding moiety makes D-serine less susceptible to disruptions of secondary structure created by expression of the NR1a subunit in isolation. In contrast, the affinity of glycine site antagonists was generally higher at NR1a homomeric NMDA receptors relative to native rat brain NMDA receptors (as characterized by Baron et al., 1996). As the antagonist molecules incorporate multiple sites for hydrophobic and hydrogen binding interactions, it follows that their affinity would be less dramatically altered by homomeric expression.

Using human homomeric NR1a receptors Grimwood et al. (1995a) have reported similar results. In their studies, agonists were much weaker competitors of [^{3}H]L-689,650 binding while antagonists had equal potencies at the homomeric NR1a and native NMDA receptors. Qualitative differences were seen among the agonists tested. For example, similar to our findings, D-serine and glycine exhibited 5.9- and 34-fold lesser affinity at homomeric vs. native receptors. As this latter study employed significantly different methodology from the present work (e.g. receptor species, host cell type, and radioligand structure) the results substantiate a fundamental difference between the properties of the glycine recognition site on the homomeric NR1a receptor relative to the native form. Taken together, these results indicate that the homomeric NR1a receptors are predominantly in an antagonist-preferring state as postulated by Monaghan et al. (1988).

4.4. Lack of allosteric interactions

Consistent with our results obtained using native receptors (Baron et al., 1996), glutamate recognition site ligands failed to modulate [^{3}H]MDL 105,519 binding to the NR1a homomeric receptor. While this may be a consequence of our demonstrated failure to obtain [^{3}H]glutamate binding to the homomeric receptor, it is also clear that allosteric effects are less prominent using [^{3}H]MDL 105,519 than in studies employing other glycine antagonists such as [^{3}H]5,7-dichlorokynurenic acid (Baron et al., 1991) or [^{3}H]L-689,650 (Grimwood et al., 1993). It should be noted that Grimwood et al. (1995a) also showed a lack of allosteric effects by unlabeled glutamate site ligands with [^{3}H]L-689,650 binding to homomeric human NR1a receptors expressed in HEK293 cells despite showing clear regulation in native receptors. These results point to a difference in the glutamate binding site in homomeric vs. native receptors and are consistent with the more detailed pharmacological characterization reported by Laurie and Seeburg (1994). These authors demonstrated [^{3}H]glutamate binding to homomeric NR1 which was resistant to displacement both by NMDA and the high affinity antagonist CGP 39653 (D,L-(*E*)-2-amino-4-propyl-5-phosphono-3-pentenoic acid). The fidelity of the heterologously expressed receptor as a model system for the native receptor must be viewed with caution since Grimwood et al. (1995b) have presented evidence that expression of the human NR1a gene in LTK mouse fibroblasts results in transport of the NR1a gene product to the cell membrane only if NMDA receptor subunit NR2A or NR2B is co-expressed.

[^{3}H]MDL 105,519 binding to the rat NR1a receptor was not affected by non-NMDA glutamatergic receptor agonists AMPA and kainate, but was comparably competed for by the AMPA receptor antagonist CNQX as seen with native NMDA receptors. Amino acid homology studies indicate the glutamate binding site of the non-NMDA receptors is homologous to the glycine binding site of the NMDA receptor (Kuryatov et al., 1994; Stern-Bach et al., 1994). The other quinoxalinedione used in this study, ACEA1021, possesses inhibitory activity at both the glycine site of the NMDA receptor (low nanomolar) and the glutamate site of the AMPA receptor (low micromolar; Woodward et al., 1995), while MDL105519 is more selective and inhibits only the glycine site of the NMDA receptor (Baron et al., 1996).

The amino acid homology of the regions that fold to create the amino acid binding site pockets of the *Salmonella typhimurium* periplasmic glutamine binding protein and the lysine-arginine-ornithine binding protein (LAOBP) is high compared to a region ~ 150 amino acids N-terminal to transmembrane I (TMI) and residues located between TMIII and TMIV of the rat (Moriyoshi et al., 1991) and human (Karp et al., 1993) NR1 subunits. Wafford et al. (1995) have demonstrated by site-directed mutagenesis that certain amino acids in these homologous regions are probably involved in binding glycine. Site-directed mutagenesis studies in the more N-terminally located putative glutamate binding site of the NR1 subunit, homologous to the binding site for *E. coli* periplasmic leucine-isoleucine-valine binding protein (LIVBP; Kuryatov et al., 1994; Stern-Bach et al., 1994) have not been reported to date. Once this region has been better defined and its tertiary structure modeled in relation to the putative glycine binding site, testable models can be proposed to better understand the allosteric interactions between these two co-agonist binding sites, if indeed there are two co-agonist binding sites located on the amino acid sequence linearly so far apart. Another possibility is that both glutamate and glycine bind in the same pocket of the NMDA receptor NR1 subunit region that is homologous to the LAOBP binding pocket. This concept is supported by pharmacological studies with CNQX and ACEA1021 which are

competitive antagonists at both the glutamate-recognition site of the AMPA receptor and the glycine-recognition site of the NMDA receptor, and a substituted isoquinoline (Ornstein et al., 1995) which is a competitive antagonist at both the glutamate-recognition site of the AMPA receptor and the glutamate-recognition site of the NMDA receptor. One chimeric receptor containing the N-terminal 400 amino acids of the NMDA-NR1 subunit (which includes the LIVBP homologous region but not the LAOBP region) and the remaining GluR6 kainate receptor subunit (which includes the LAOBP homologous region) has been reported to have similar affinity for glutamate as the wild type GluR6 subunit (Stern-Bach et al., 1994), possibly giving evidence that the LIVBP region of the NMDA receptor NR1 subunit is not the binding region for the agonist glutamate. Truncated NMDA-NR1a receptors similar to one recently reported for the AMPA receptor GluR4 (Kuusinen et al., 1995) might help to define the glutamate and glycine co-agonist binding pockets of the NMDA receptor. Homomeric NR1 receptors and heteromeric NMDA receptors of defined subunit composition, along with radioligands such as [^{3}H]MDL 105,519 should prove to be useful tools for such further study.

In summary, the comparison of the radioligand binding studies using NR1a homomeric receptors with those of native receptors suggests differences in the characteristics of both the glutamate and glycine recognition sites and their allosteric interactions. These results may be the consequence of an absence of some of the co-agonist site components, improper folding, or structural barriers to the interaction of two co-agonist sites.

Acknowledgements

We thank Professor Shigetada Nakanishi for the generous gift of plasmid pN60 which contains the rat NR1a gene; Dr Mamata De and Ms Kimberly Austin for the CHO-K1 cell line and detailed procedure for transfection by electroporation; and Dr Keith Kropp for advice on expression vector construction and selection of stable transfections. We are also grateful to Dr Carrie Markgraf for help and advice in statistical analyses.

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