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Short communication

[^{3}H]MDL 105,519 binds with equal high affinity to both assembled and unassembled NR1 subunits of the NMDA receptor

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Abstract

[^{3}H]MDL 105,519 (((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1[^{3}H]-indole-2-carboxylic acid) is a novel radioligand which binds with high affinity, $K_d = 2.5$ nM, to the glycine site of adult rodent forebrain, *N*-methyl-D-aspartate subtype of glutamate receptors. As with other glycine site antagonists, the major determinants for high-affinity binding of [^{3}H]MDL 105,519 resides upon the NR1 subunit, and not the NR2 subunits. [^{3}H]MDL 105,519 binds with equal affinity, $K_d = 3$ nM, to both NR1-1a or NR1-4b splice variants, as well as the NR1-1a/NR2A receptor expressed in human embryonic kidney (HEK) 293 cells. One percent Triton X-100/1 M NaCl solubilises with a recovery of 15 ± 3%, a mixed pool of assembled and unassembled forebrain NR1 subunit polypeptides. In this preparation, the recovery of [^{3}H]MK801 ((+)-5-[^{3}H]methyl-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5,10-imine binding activity (7 ± 1%) reflects the amount of assembled NR1 subunits whereas [^{3}H]MDL 105,519 binds quantitatively, with a recovery of 19 ± 4% and $K_d = 3$ nM, to both assembled and unassembled NR1 subunits. Therefore, [^{3}H]MDL 105,519 should prove a useful ligand, in conjunction with immunopurification approaches, to address the question of NMDA receptor subunit stoichiometry.

Keywords: NMDA receptor; Glutamate receptor; Glycine; Triton X-100; NR1

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is a ligand-gated cation channel which is widely expressed throughout the mammalian central nervous system. A feature of this receptor is that receptor activation requires the binding of both the agonist, glutamate, and the co-agonist, glycine, to discrete but allosterically coupled binding sites, thus providing, uniquely, two primary sites for pharmacological intervention. Recently, several novel, high-affinity glycine site antagonists have been described including L-689,560 ((±)-4-(*trans*)-2-carboxy-5,7-dichloro-4-phenylaminocarbonylaminino-1,2,3,4-tetrahydroquinoline) (Grimwood et al., 1995) and MDL 105,519 (((*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloroindole-2-carboxylic acid) (Baron et al., 1996; Siegel et al., 1996), thus providing new tools for NMDA receptor characterisation and localisation by autoradiographic studies. Molecular cloning has identified five genes encoding two types of NMDA receptor subunit, the NR1 subunit gene which undergoes alternative splicing to yield eight variant NR1 subunits, NR1-1a,b to NR1-4a,b and the NR2A–D subunit genes (McBain and Mayer, 1994). Functional native NMDA receptors are believed to be heteromeric complexes comprising both NR1 and NR2 subunits with different pharmacological and biophysical properties. In order to determine the subunit compositions of native NMDA receptors, we have recently described their immunoaffinity purification from mammalian forebrain (Chazot and Stephenson, 1997a,b). During the course of these studies, we found evidence for a significant pool of unassembled NR1 subunits expressed in adult rodent brain (Chazot and Stephenson, 1997a). Since the glycine binding site has been localised to the NR1 subunit, it may be that significant radioactive glycine site antagonist binding is to non-functional, monomeric NR1 subunits. In this paper, we have characterised the radioligand binding properties of the novel glycine site antagonist, [^{3}H]MDL 105,519, to cloned NMDA receptor subtypes and native, membrane-bound and Triton X-100-solubilised NMDA receptors. We provide evidence that, indeed, glycine site antagonists do bind to native, unassembled NR1 subunits.

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2. Materials and methods

2.1. Materials

[^{3}H]MDL 105,519 (74 Ci mmol^{-1}) was from Amersham International (Aylesbury, Bucks., UK) and (+)-5-[^{3}H]methyl-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5,10-imine ([^{3}H]MK801; 28.8 Ci mmol^{-1}) was from Du Pont (UK) (Stevenage, Hertfordshire, UK). The MK801 was from Research Biochemicals (Natick, MA, USA). The 5,7-dichlorokynurenic acid and 7-chlorokynurenic acid were from Tocris Neuramin (Bristol, UK). NMDA receptor cDNAs were generous gifts from Drs. S. Nakanishi (Kyoto, Japan) and M. Mishina (Nigata, Japan). Anti-NR1 17–35 antibodies were prepared as previously described (Chazot et al., 1995). All other materials were from commercial sources.

2.2. Membrane preparations

Well-washed P2 membranes were prepared from adult Wistar rat forebrains as previously described (Chazot et al., 1993). Cell homogenates were prepared from transfected human embryonic kidney (HEK) 293 cells as before (Chazot et al., 1994). Briefly, NR1-1a, NR1-4b, NR2A, NR2B and NR2C NMDA receptor cDNAs were subcloned into the mammalian expression vector, pCIS. The HEK 293 cells were transfected with each individual plasmid (10 μg DNA) or NR1-1a/NR2A, NR1-1a/NR2B binary combinations (10 μg DNA in a 1:3 ratio, respectively) using the calcium phosphate precipitation procedure. Cells were harvested 24 h post-transfection, cell suspensions were centrifuged at $3000 \times g$ for 5 min at 4°C, the cell pellet collected and homogenised with a glass/glass homogeniser for 10 strokes in ice-cold 50 mM Tris, pH 7.4, containing 5 mM EDTA and 5 mM EGTA. The homogenate was recentrifuged at $30\,000 \times g$ for 30 min at 4°C. The cell homogenate was rehomogenised, centrifuged and the final cell pellet resuspended and assayed immediately for [^{3}H]MDL 105,519 radioligand binding activity. The NMDA receptor subunit expression in the transfected cells was verified by immunoblotting using the appropriate anti-NMDA receptor subunit-specific antibody (e.g., Chazot and Stephenson, 1997a).

2.3. Solubilisation of adult rat forebrain membranes

Rat forebrain P2 membranes were resuspended to a protein concentration of 1.5 mg ml^{-1} in 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylmethylsulphonyl fluoride, bacitracin (5 μg ml^{-1}), benzamidine HCl (5 μg ml^{-1}), soybean trypsin inhibitor (5 μg ml^{-1}), and ovomucoid trypsin inhibitor (5 μg ml^{-1}). Triton X-100 and NaCl were added to final concentrations of 1% (v/v) and 1 M, respectively and the extract stirred gently at 4°C for 30 min. The soluble extract was collected by centrifugation at $100\,000 \times g$ for 30 min at 4°C. The pellet was resuspended and washed with 2×15 ml 50 mM Tris–HCl, pH 7.5, containing 5 mM EDTA and 5 mM EGTA and assayed for [^{3}H]MDL 105,519 radioliogand binding activity. Gel filtration was performed essentially as described by Chazot and Stephenson (1997a), except that elution was carried out in 50 mM Tris, pH 7.5, containing 0.05% (v/v) sodium deoxycholate.

2.4. Radioligand binding

[^{3}H]MK801 radioligand binding assays were performed as described previously in the presence of 10 μM glutamate and 10 μM glycine, using a polyethylenimine filtration assay, with 10 μM MK801 for the determination of non-specific binding and an incubation time of 24 h at 4°C (Chazot and Stephenson, 1997a). [^{3}H]MDL 105, 519 binding assays were carried out using a polyethyleimine filtration assay with 100 μM glycine for the determination of non-specific binding and an incubation time of 90 min at 4°C. Dissociation constants were determined by either the measurement of the rates of association and dissociation constants (native NMDA receptors) or by direct saturation radioligand binding (cloned NMDA receptors). For displacement studies, 0.5 nM [^{3}H]MDL 105,519 concentrations, respectively, were used. For the assay of both [^{3}H]MK801 and [^{3}H]MDL 105,519 Triton X-100-solubilised binding activities, soluble samples (1 ml) were firstly detergent-exchanged by gel filtration using a Sephadex G-25 column (1×10 cm), equilibrated and eluted with 0.05% (v/v) sodium deoxycholate, since both 1% Triton X-100 and 1 M NaCl were found to inhibit [^{3}H]MK801 and [^{3}H]MDL 105,519 radioligand binding activities. Fractions of 1 ml were collected at a rate of 10 ml h^{-1} at 4°C. Receptor-containing fractions (2 ml) were pooled and assayed for radioligand binding activities as above. Binding data were analysed by Inplot (Graphpad).

3. Results

3.1. [^{3}H]MDL 105,519 radioligand binding to native membrane-bound NMDA receptors

In preliminary studies, a time course of specific [^{3}H]MDL 105,519 radioligand binding activity was carried out to well-washed, adult rat forebrain membranes. Specific [^{3}H]MDL 105,519 binding activity reached equilibrium after 60 min and remained stable for at least 3 h at 4°C. Both the association and dissociation rates were best fitted to a single exponential with $k_{+1} = 3.2 \pm 0.2 \times 10^7$ M^{-1} min^{-1}, $t_{1/2} = 10 \pm 3$ min and $k_{-1} = 0.080 \pm 0.008$ min^{-1}, respectively (results not shown). This gave a K_d value of 2.5 nM ± 0.3 nM (mean ± S.D., $n = 3$ independent determinations). The pharmacological specificity of [^{3}H]MDL 105,519 binding was determined by displace-

Table 1
Pharmacological specificity of [^{3}H]MDL 105,519 radioligand binding to adult rat forebrain membranes

Compound	K_i (nM)	Hill slope (n_H)
Glycine	580 ± 370	0.80 ± 0.02
D-Serine	750 ± 360	1.07 ± 0.03
L-Serine	NE	ND
L-701,324	8.4 ± 3.9	0.84 ± 0.09
5,7-Dichlorokynurenic acid	82 ± 9	1.03 ± 0.03
7-Chlorokynurenic acid	700 ± 200	0.98 ± 0.21
Glutamate	NE	ND
AP5	NE	ND

Competition experiments were performed by a radioligand binding filtration assay using 0.5 nM [^{3}H]MDL 105,519 as described in Section 2. The resultant displacement curves were fitted using Inplot (Graphpad) and all were best fit by a single site. Results are mean ± S.D. for three separate determinations each performed in triplicate.
ND = not determined.
NE = no effect at 10^{-4} M.

ment studies with a range of known glycine site ligands. The results are summarised in Table 1. All the resultant displacement curves were best fit to a single binding site model with Hill coefficients close to unity (Table 1). The rank order of potency was L-701,324 > 5,7-dichlorokynurenic acid > 7-Cl-kynurenic acid = glycine = D-serine ⋙ L-serine. The stereospecificity for the agonist, serine, was maintained with the D-isomer being at least two orders of magnitude more potent that the L-isomer (Table 1). Under equilibrium conditions, neither glutamate nor DL-2-amino-5-phosphonopentanoic acid (AP5) at a maximum concentration of 10^{-4} M had a significant effect upon [^{3}H]MDL 105,519 binding activity.

3.2. [^{3}H]MDL 105,519 binding to cloned NMDA receptors expressed in HEK 293 cells

Initially, [^{3}H]MDL 105,519 radioligand binding was carried out to the NR1-1a, NR1-4b, NR2A, NR2B and NR2C subunits each expressed alone in HEK 293 cells. Specific binding was found to the NR1-1a and NR1-4b subunits only. Specific [^{3}H]MDL 105,519 binding to the singly expressed NR1-1a and NR1-4b subunits was saturable with dissociation constants (K_d values) 3.2 ± 1.9 nM and 2.7 ± 2.0 nM, respectively. Table 2 summarises the K_d values determined where it can be seen that no significant difference was found for the binding of [^{3}H]MDL 105,519 to native forebrain NMDA receptors, NR1-1a and NR1-4b expressed alone and the binary NR1-1a/NR2A subunit combination.

Table 2
Dissociation constants for the binding of [^{3}H]MDL 105,519 to native and cloned NMDA receptor preparations

Preparation	Dissociation constant (nM)	*n*
Forebrain membranes	2.5 ± 0.3	3
Triton X-100-solubilised forebrain membranes	2.8 ± 1.0[a]	3
NR1-1a/NR2A	1.9 ± 1.0[a]	2
NR1-1a	3.2 ± 1.9[a]	3
NR1-4b	2.7 ± 2.0[a]	4

The dissociation constants were determined by a non-transformed curve fitting of saturation binding assays using [^{3}H]MDL 105,519, performed as described in Section 2. Data were best fit to a single binding site model using Inplot (Graphpad). Results are means ± S.D. for between two and four separate determinations.
[a] Values not significantly different from membranes ($P < 0.001$).

3.3. [^{3}H]MDL 105,519 binding to NMDA receptors solubilised from rat forebrain with 1% Triton X-100 / 1 M NaCl

We have previously shown that solubilisation of mammalian forebrain membranes with 1% Triton/1 M NaCl at 4°C extracted 15 ± 3% of the total anti-NR1 subunit immunoreactivity with the recovery of 7 ± 2% of the initial [^{3}H]MK801 binding activity in the membrane fraction (Chazot and Stephenson, 1997a). The difference between these two values can be explained by the fact that in the Triton X-100-solubilised preparation, 45% of the NR1 subunits existed as unassembled monomers; [^{3}H]MK801 has been shown to bind with high affinity to assembled NMDA receptors (Chazot et al., 1994). In contrast, Triton X-100 solubilised 19 ± 4% of the [^{3}H]MDL 105,519 binding sites present in the original membrane fraction. This value corresponds to that found for the efficiency of solubilisation of anti-NR1 immunoreactivity by Triton X-100, i.e., 15 ± 3%. Based on gel filtration, the proportion of solubilised [^{3}H]MDL 105,519 binding sites were 53 ± 1% and 47 ± 1% for the oligomeric and monomeric peaks, respectively, values which are consistent with the relative NR1 subunit immunoreactivities reported previously (Chazot and Stephenson, 1997a). [^{3}H] MDL 105,519 radioligand binding to the Triton X-100-solubilised preparation after sodium deoxycholate detergent-exchange was saturable and best fit by a single high affinity site with K_d = 2.8 nM. This value is not significantly different to that found for the binding of [^{3}H]MDL 105,519 to membrane-bound native NMDA receptors and also, single and binary NMDA receptor subunit combinations (Table 2).

4. Discussion

In this paper, we have described the use of the novel glycine site antagonist, [^{3}H]MDL 105,519 to study the molecular properties of native and recombinant NMDA receptor subtypes. It was shown that [^{3}H]MDL 105,519 binds to a single high-affinity site, K_d = 2.5 nM, in adult rat forebrain membranes with a pharmacological profile that is consistent with that of the glycine site of the NMDA receptor. Furthermore, [^{3}H]MDL 105,519 binds with similar affinity to both NR1-1a and NR1-4b monomers

and NR1-1a/NR2A heteromeric NMDA receptors, thus demonstrating firstly, that the determinants for high affinity [^{3}H]MDL 105,519 binding reside within the NR1 subunit and, secondly that the N1 exon of the NR1 subunit, present in the NR1-4b splice form, does not affect [^{3}H]MDL 105,519 binding affinity. These results confirm but also extend the studies by Siegel et al. (1996) who first reported the binding of [^{3}H]MDL 105,519 to cloned NR1-1a subunits. Further, these results are consistent with other classes of glycine site antagonists including 5,7-dichlorokynurenic acid and L-689,560, which have also been shown to bind with high affinity to the NR1 subunit when expressed alone in mammalian cells (e.g., Laurie and Seeburg, 1994; Lynch et al., 1993; Grimwood et al., 1995).

A prerequisite for biochemical and purification studies is to extract receptors efficiently and in a stable form from the membrane. One solubilisation protocol utilised recently is 1% Triton X-100/1 M NaCl, which we have shown extracts both assembled and non-assembled NMDA receptors from mammalian forebrain. The number of [^{3}H]MK801 binding sites correlates well with the amount of assembled NR1 subunit protein in the soluble extract, while the number of [^{3}H]MDL 105,519 binding sites correlates well with the amount of total NR1 subunit protein. Solubilisation had no significant effect upon the affinity of [^{3}H]MDL 105,519 binding. This feature distinguishes [^{3}H]MDL 105,519 binding to the NMDA receptor from [^{3}H]AMPA binding to non-NMDA receptors, where a significant increase in apparent binding affinity is observed upon extraction with Triton X-100 (e.g., Hunter and Wenthold, 1992).

5. Conclusion

We have demonstrated by two independent approaches that [^{3}H]MDL 105,519 selectively and quantitatively labels both assembled heteromeric and unassembled monomeric membrane-bound cloned and detergent-solubilised native NR1 subunits, which highlights the need for caution when interpreting autoradiographical information using this class of NMDA receptor ligand (Laurie and Seeburg, 1994). However, in conjunction with an immunopurification approach, [^{3}H]MDL 105,519 should be a useful ligand to address the question of native NMDA receptor subunit stoichiometry.

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