ELSEVIER

Molecular Brain Research 73 (1999) 189–192

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Short communication

A mutation in the glycine binding pocket of the *N*-methyl-D-aspartate receptor NR1 subunit alters agonist efficacy

Michael W. Wood, Hendrika M.A. VanDongen, Antonius M.J. VanDongen *

Department of Pharmacology and Cancer Biology, Duke University Medical Center, PO Box 3813, Durham, NC 27710, USA

Accepted 27 July 1999

Abstract

Alanine 714 of the NMDA receptor NR1 subunit resides in the glycine binding pocket. The Ala714Leu mutation substantially shifts glycine affinity, but here no effect on antagonism by DCK is detected. Ala714Leu is also found to limit the efficacy of a partial agonist without altering its apparent affinity. The differential sensitivity of Ala714Leu to glycine agonists suggests that alanine 714 may be an intermediary in transducing the ligand binding signal. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: NMDA receptor; Pharmacology; Efficacy; Affinity; DCK; HA-966

Glycine and L-glutamate are both required to activate the NMDA receptor [6,7,12]. Although the relationship between these two coagonists is complex [10,17], much progress has been made in defining the binding sites of L-glutamate and glycine at the molecular level. A current image of the NMDA receptor assigns each of the binding sites to separate subunit members. L-glutamate activation is disrupted by mutations in NR2 subunits [9], while numerous studies of mutated residues in the NR1 subunit demonstrate disturbances in the apparent affinity for the coagonist glycine [5,8,20,23]. The extracellular ligand-binding portions of each subunit member are thought to exist in a bilobate conformation [1,16], with the first lobe (S1) represented by portions of the amino terminus, and the second lobe (S2) composed largely of the region connecting the M3 and M4 transmembrane domains. The recent crystallization of a fusion protein constructed from a homologous receptor (i.e., iGluR2) offers a high resolution structural image of this model [1].

We previously found that a quadruple mutation (N710R, Y711R, E712A, A714L) in the M3–M4 loop of the NR1 subunit disrupts glycine binding [23]. Dichlorokynurenic acid (DCK) is a high affinity competitive antagonist at the glycine site of the NMDA receptor [4,13]. When measured near their respective EC_{50}s for glycine (see Table 1), analysis of DCK affinity revealed no difference between the wild type and quadruple mutant receptors (both IC_{50}s $= 0.3$ μM, see Fig. 1 and Table 1). Application of the Cheng–Prussoff equation led to indistinguishable estimates of the affinity constants for DCK as well (K_is $= 0.16$ μM, Table 1).

The residue dominant in conferring the shift in glycine affinity in the quadruple mutant was the alteration of alanine 714 to leucine [23]. Although the mutation Ala714Cys did not induce a shift in the apparent affinity for glycine, it was acutely sensitive to alteration by the thiol-modifying reagent, dithionitrobenzoic acid (DTNB). Co-application of glycine, but not L-glutamate, with DTNB could protect A714C from modification by DTNB (see Fig. 7 of Wood, et al. [23]). Unlike glycine, DCK was not able to protect A714C from inhibition by DTNB (Fig. 2). Thus, there is no evidence supporting the involvement of alanine 714 in binding of DCK.

(±)-3-Amino-1-hydroxy-2-pyrrolidone (HA-966) has been used as a partial agonist at the glycine site of the NMDA receptor [17–19]. We performed a comparative analysis of the wild type receptor and the quadruple mutant receptor employing HA-966 as a partial agonist (Fig. 3). Although no significant difference in affinity was seen between the wild type and mutant receptors, the quadruple mutant displayed markedly reduced maximal activation (i.e., efficacy) by HA-966 (Table 2). HA-966 affinity and efficacy were examined for the individual point mutants composing the quadruple mutant. The A714L mutant displayed the most prominent reduction in HA-966 efficacy (Fig. 3C and Table 2).

* Corresponding author. Fax: +1-919-684-8922; e-mail: vando005@mc.duke.edu

PII: S0169-328X(99)00247-8

Table 1
DCK affinity

Construct	[Gly] (μM)	IC50 (μM)	(95% C.I.)	nH	(95% C.I.)	Gly EC50 (μM)[a]	Kb (μM)[b]
NR1	1.5	0.31	0.15–0.64	1.67	0.7–2.6	1.6	0.16
N710R, Y711R, E712A, A714L	500	0.32	0.21–0.48	0.99	0.7–1.3	530	0.16

[a] Taken from Wood et al. [23].
[b] Derived from relationship described by Cheng and Prussoff, 1973. (See D.A. Craig, TIPS 141 (1993) 89–91).

Recently, a fusion protein forming a glutamate-receptor ligand-binding core was crystallized. In that structure, the residue analogous to A714 of NR1 is threonine 686 of iGluR2 [1]. T686 resides within the binding pocket for the agonist in the S2 lobe (i.e., kainate in the iGluR2 S1S2 crystal). However, T686 is not a contact point for kainate, but instead forms a hydrogen bond with E402 in the S1 lobe, thereby stabilizing the agonist-bound conformation. In previous work, we also concluded that A714 resided within the glycine-binding pocket of the NMDA receptor, but did not represent a contact point for glycine [23].

The major difficulty in interpreting changes in apparent affinity following mutation of a receptor is distinguishing between a mutation that affects agonist binding (affinity) from a mutation that affects transduction of the binding signal (efficacy). For a careful review of this issue, see Colquhoun [2]. In the case of a ligand-gated ion channel, the simplest model describes three states of the receptor/channel: unoccupied, agonist-occupied/inactive, and agonist-occupied/active. If a mutation affects the binding of agonist, it alters the first equilibrium (i.e., K_A); if it affects transduction, it alters the second equilibrium (i.e., E). However, for a highly efficacious agonist possessing a large E, a mutation that alters the second equilibrium will appear to affect the affinity by causing a shift in

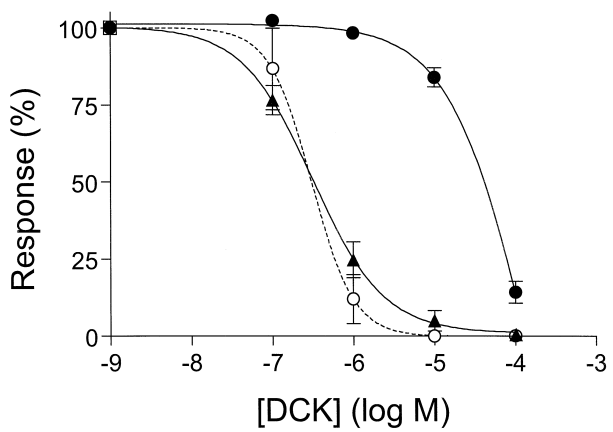

Fig. 1. The quadruple NR1 mutation (710:NYEA → RRAL) does not alter DCK affinity. Mutagenesis of NR1 (a kind gift of Dr. S. Nakanishi [15]) and synthesis of in vitro cRNA was described previously [23]. The ε1 clone (mouse NR2A, a kind gift of Dr. M. Mishina [14]) was coinjected into *Xenopus laevis* oocytes with NR1 constructs with measurements made 2–5 days later [22]. Whole cell agonist responses were conducted as previously described [21]. Oocytes were held at −50 mV and perfused continuously with a Mg^{2+}- and Ca^{2+}-free solution containing (in mM): 100 NaCl, 5 KCl, 0.5 $BaCl_2$, and 10 HEPES, pH 7.4, 25°C. Peak current responses and accompanying voltage measurements were determined using PCLAMP hardware and software (Axon Instruments, Burlingame, CA). Effective concentration at 50% (EC_{50}) and inhibitory concentrations at 50% (IC_{50}) values were calculated using the Graph Pad software package (Graph Pad Software). All values are reported with the 95% confidence intervals generated by the Graph Pad models. DCK inhibition was assessed in oocytes expressing either the NR1 + NR2A (wild type) or the quadruple mutation of NR1 + NR2A combination. The L-glutamate concentration was fixed at 100 μM for all experiments. Glycine concentrations were fixed near the previously determined EC_{50} values: 1.5 μM for the wild type (open circles and dashed line, $n = 3$), 500 μM for the mutant (filled triangles, $n = 5$) combinations. Measurements of the wild type combination were also performed at 500 μM glycine (filled circles, $n = 6$). Initial responses to agonist (L-glutamate + glycine) were determined. These were followed by measurements at test concentrations of DCK and a final agonist-only measurement. DCK inhibition was calculated by normalizing responses that included DCK to responses preceding and following the inclusion of antagonist. Mean responses and resulting dose–response curves are shown. Error bars represent standard errors and are omitted when smaller than the symbol.

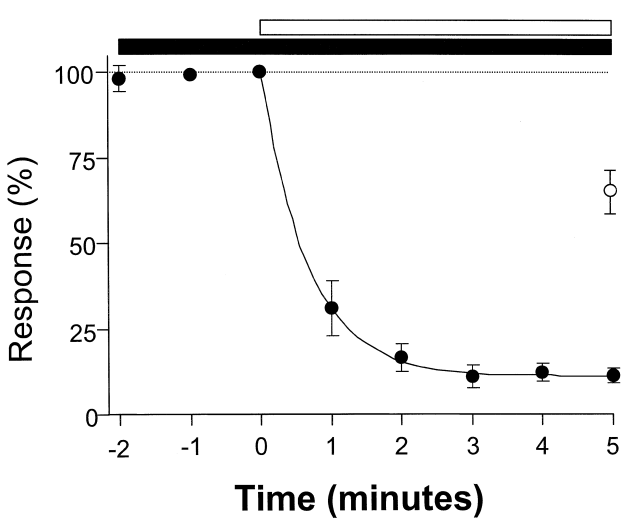

Fig. 2. DCK does not protect NR1-A714C from DTNB modification. Initial responses to 100 μM L-Glu + 100 μM Gly + 100 μM DCK (black bar) were assessed in oocytes expressing the NR1-A714C mutant + NR2A subunits. After stabilization of response, oocytes were exposed to continuous application of 500 μM DTNB (white bar). The mean and SEM of the responses to agonist are represented by the filled squares ($n = 3$). The means were fit by a single exponential ($\tau = 27$ s, plateau = 11%). The open square displays the degree of protection offered by 100 μM glycine after 5 min of exposure to 500 μM DTNB in a similar experiment (see Ref. [23]).

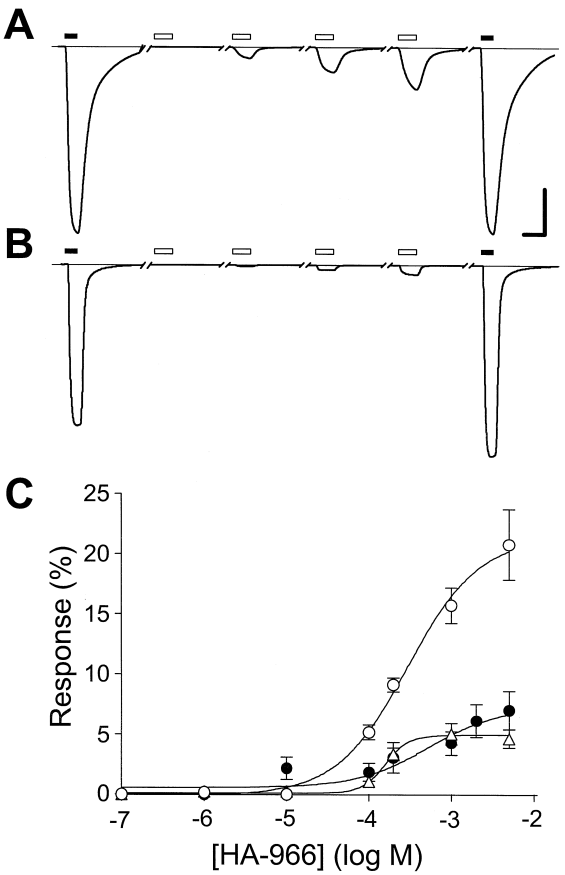

Fig. 3. Activation by HA-966 is diminished in the quadruple mutation of NR1. Representative traces of whole cell responses of the wild type receptor (A) and quadruple mutation of NR1 (B) are shown. Normalizing concentrations of agonist (100 μM L-Glu + 100 μM Gly) are shown as black bars with test responses to increasing concentrations of HA-966 (10 μM, 100 μM, 2 mM, and 5 mM) + 100 μM L-Glu shown as open bars. Scale bars represent 10 s and 1 μA for the wild type receptor and 2 μA for the quadruple mutant. (C) Dose–response curves for the wild type (open circles), quadruple NR1 mutant (open triangles), and the NR1-A714L mutant (closed circles) are shown. Symbols represent mean responses calculated as in Fig. 1. Error bars represent standard errors.

the EC50 [2]. The A714L mutation did reduce the apparent affinity for the full agonist glycine, as measured by the EC50 [23]. Now, using a less efficacious agonist (i.e., HA-966), we find no change in affinity for a partial agonist and significant reduction in the efficacy as a result of the A714L mutation. Such a phenotype is predicted for a transduction mutant [2]. The lack of effect on antagonism by DCK further supports the idea that this region is not involved in directly binding glycine.

This pharmacological profile described thus far suggests that the role of A714 in NR1 may be the transduction step of NMDA receptor activation. A714 of NR1 is equivalent to residue T686 in iGluR2. T686 is also found within the ligand binding site and forms a hydrogen bond with E402 in the agonist-bound conformation [1]. Thus, it is likely that T686 of iGluR2 stabilizes the agonist-occupied/active conformation of the receptor, and thereby promotes channel opening. So, although residue T686 is not a contact point for glutamate, it may contribute to the molecular determinants for agonist efficacy in iGluR2. Based on the data presented here, we propose that the domain containing A714 in NR1 also contributes to agonist efficacy by stabilizing the agonist-bound conformation.

The necessity for two agonists (L-glutamate and glycine) increases the theoretical complexity of NMDA receptor activation [3,11]. The mechanics that underlie subunit interactions are also unknown. These limitations narrow our ability to interpret mutagenesis data. However, despite these limitations, the data presented here contribute to understanding the pharmacological characteristics of the NMDA receptor. Moreover, this type of analysis in conjunction with the static views generated from crystal structures may assist in developing a dynamic image of a functioning receptor.

Acknowledgements

This work was supported by Grant NS31557 from the National Institute of Neurological Disorders and Stroke (to A.M.J.V.D.). M.W.W. was supported by National Research Service Award MH11278 from the National Institute of Mental Health.

Table 2
Apparent affinities for glycine and HA-966

Construct	HA-966 affinity	(95% C.I.)	Alpha (%)	*n*	Glycine affinity (μM)[a]
NR1	298	200–450	21 ± 2.4	11	1.6
N710R, Y711R, E712A, A714L	159	110–230	4.9 ± 0.8	6	530
N710R	149	40–520	24 ± 7	6	3.5
Y711R	122	44–340	23 ± 6	5	3.6
E712A	78	20–310	22 ± 6	5	4.3
A714L	532	120–2300	7 ± 2.5	9	100

[a] From Wood et al. [23].

References

[1] N. Armstrong, Y. Sun, G.Q. Chen, E. Gouaux, Structure of a glutamate-receptor ligand-binding core in complex with kainate, Nature 395 (1998) 913–917.

[2] D. Colquhoun, Binding, gating, affinity and efficacy — the interpretation of structure-activity relationships for agonists and the effect of mutating receptors, Br. J. Pharmacol. 125 (1998) 924–947.

[3] M. Corsi, P. Fina, D.G. Trist, Co-agonism in drug-receptor interaction: illustrated by the NMDA receptors, Trends Pharmacol. Sci. 17 (1996) 220–222.

[4] A.C. Foster, J.A. Kemp, P.D. Leeson, S. Grimwood, A.E. Donald, G.R. Marshall, T. Priestley, J.D. Smith, R.W. Carling, Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the *N*-methyl-D-aspartate receptor from rat brain, Mol. Pharmacol. 41 (1992) 914–922.

[5] H. Hirai, J. Kirsch, B. Laube, H. Betz, J. Kuhse, The glycine binding site of the *N*-methyl-D-aspartate receptor subunit NR1: identification of novel determinants of co-agonist potentiation in the extracellular M3–M4 loop region, Proc. Natl. Acad. Sci. USA 93 (1996) 6031–6036.

[6] J.W. Johnson, P. Ascher, Glycine potentiates the NMDA response in cultured mouse brain neurons, Nature 325 (1987) 529–531.

[7] N.W. Kleckner, R. Dingledine, Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes, Science 241 (1988) 835–837.

[8] A. Kuryatov, B. Laube, H. Betz, J. Kuhse, Mutational analysis of the glycine-binding site of the NMDA receptor: structural similarity with bacterial amino acid-binding proteins, Neuron 12 (1994) 1291–1300.

[9] B. Laube, H. Hirai, M. Sturgess, H. Betz, J. Kuhse, Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding sites on the NR2B subunit, Neuron 18 (1997) 493–503.

[10] R.A.J. Lester, Tong, C.E. Jahr, Interactions between the glycine and glutamate binding sites of the NMDA receptor, J. Neurosci. 13 (1993) 1088–1096.

[11] J.-C. Marvizon, M. Baudry, Receptor activation by two agonists: analysis by nonlinear regression and application to *N*-Methyl-D-Aspartate receptors, Anal. Biochem. 213 (1993) 3–11.

[12] M.L. Mayer, L. Vyklicky Jr., J. Clements, Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine, Nature 338 (1989) 425–427.

[13] D. McNamara, E.C.R. Smith, D.O. Calligaro, P.J. O'Malley, L.A. McQuaid, R. Dingledine, 5,7-Dichlorokynurenic acid, a potent and selective competitive antagonist of the glycine site on NMDA receptors, Neurosci. Lett. 120 (1990) 17–20.

[14] H. Meguro, H. Mori, K. Araki, E. Kushiya, T. Kutsuwada, M. Yamazaki, T. Kumanishi, M. Arakawa, K. Sakimura, M. Mishina, Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs, Nature 357 (1992) 70–74.

[15] K. Moriyoshi, M. Masu, T. Ishii, R. Shigemoto, N. Mizuno, S. Nakanishi, Molecular cloning and characterization of the rat NMDA receptor, Nature 354 (1991) 31–37.

[16] P.J. O'Hara, P.O. Sheppard, H. Thogersen, D. Venezia, B.A. Haldeman, V. McCrane, K.M. Houamed, C. Thomsen, T.L. Gilbert, E.R. Mulvihill, The ligand binding domain in metabotropic glutamate receptors is related to bacterial periplasmic binding proteins, Neuron 11 (1993) 41–52.

[17] T. Priestley, J.A. Kemp, Kinetic study of the interactions between the glutamate and glycine recognition sites on the *N*-methyl-D-aspartic acid receptor complex, Mol. Pharmacol. 46 (1994) 1191–1196.

[18] T. Priestley, P. Laughton, J. Myers, B. Le Bourdelles, J. Kerby, P.J. Whiting, Pharmacological properties of recombinant human *N*-methyl-D-aspartate receptors comprising NR1a/NR2A and NR1a/NR2B subunit assemblies expressed in permanently transfected mouse fibroblast cells, Mol. Pharmacol. 48 (1995) 841–848.

[19] L.M. Pullan, R.J. Powel, R.J. Stumpo, M. Britt, A.B. Klika, B.A. Meiners, A.I. Salama, Stereoselective enhancement by (*R*)-HA-966 of the binding of [3H]CPP to the NMDA receptor complex, Eur. J. Pharmacol. 189 (1990) 237–240.

[20] K. Williams, J. Chao, K. Kashiwagi, T. Masuko, K. Igarashi, Activation of *N*-methyl-D-aspartate receptors by glycine: role of an aspartate residue in the M3–M4 loop of the NR1 subunit, Mol. Pharmacol. 50 (1996) 701–708.

[21] M.W. Wood, H.M.A. VanDongen, A.M.J. VanDongen, Structural conservation of ion conduction pathways in K channels and glutamate receptors, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4882–4886.

[22] M.W. Wood, H.M.A. VanDongen, A.M.J. VanDongen, The 5′-untranslated region of the *N*-methyl-D-asparate receptor NR2A subunit controls efficiency of translation, J. Biol. Chem. 271 (1996) 8115–8120.

[23] M.W. Wood, H.M.A. VanDongen, A.M.J. VanDongen, An alanine residue in the M3–M4 linker lines the glycine binding pocket of the *N*-Methyl-D-Aspartate receptor, J. Biol. Chem. 272 (1997) 3532–3537.