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Glycine–glutamate interactions at the NMDA receptor: role of cysteine residues

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The NMDA subtype of ionotropic glutamate receptors requires occupation by both L-glutamate and the co-agonist glycine for efficient channel opening. To elucidate the role of disulfide bridges for the allosteric interaction of these agonists we mutated the cysteine residues in the ligand-binding NMDAR1 (NR1 or ζ) subunit of the rodent NMDA receptor and co-expressed the resulting mutants with the NR2B (ε2) subunit in *Xenopus* oocytes. Most of the cysteine substitutions had no effect on agonist responses. However, replacement of cysteines 402 and 418 by alanine largely abolished the potentiation of glutamate currents by glycine. These cysteine residues in the putative extracellular domain of the NR1 subunit may form a disulfide bridge important for agonist interaction.

Glutamate receptor; NMDA receptor; Agonist binding; Glycine potentiation; Site-directed mutagenesis

1. INTRODUCTION

The amino acid glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. Its postsynaptic actions are mediated by a diverse set of specific receptors, which have been implicated in brain development, synaptic plasticity, memory formation and neurotoxicity [1–3]. Amongst these, the *N*-methyl-D-aspartate (NMDA) receptor has gained particular attention, since this ligand-gated ion channel is highly permeable to Ca^{2+} ions and blocked in a voltage-dependent manner by Mg^{2+} ions [4]. These properties are thought to provide the molecular basis for activity-dependent adaptation mechanisms including long-term potentiation and heterosynaptic depression [5]. In addition, both glutamate and glycine are required for efficient channel activation of the NMDA receptor [6,7]. This dual regulation of NMDA receptor function underlines the role of this membrane protein as a postsynaptic coincidence detector [1,2] and may be important for the modulation of synaptic efficacy [2,5].

The first cDNA encoding a NMDA receptor protein was isolated by expression cloning [8]. When singly expressed in *Xenopus* oocytes, this NMDAR1 (NR1 or ζ; see [8–11] for nomenclature) subunit generates channels, which display most of the typical pharmacology of the native NMDA receptor. In particular, significant current responses are only seen upon co-application of glutamate and glycine, and these currents are readily blocked by Mg^{2+} ions [8,9]. However, the maximal current responses obtained from these homo-oligomeric NR1 channels are small, but strongly increase upon co-expression of a member of a second family of NMDA receptor proteins, the NR2 (or ε) subunits [10–13]. The NR2 subunits do not generate detectable currents upon single expression, but strongly potentiate NR1 responses upon co-expression. Based on these data, the NR1 and NR2 subunits can be classified as ligand-binding and structural subunits, respectively, of the mammalian NMDA receptor.

Pharmacological and electrophysiological studies indicate that glutamate and glycine bind to distinct sites of the NMDA receptor [6,7,14,15]. The mechanism of glycine potentiation of glutamate currents is not entirely clear, but may involve allosteric modulation of agonist binding affinities in addition to changes in desensitization kinetics [16,17]. To elucidate the molecular basis of these agonist interactions, we performed in vitro mutagenesis of the ligand-binding NR1 subunit. Here, we show that two cysteine residues, cysteine 402 and cysteine 418, are important for the potentiation of glutamate currents by glycine. Our data suggest that a disulfide bridge formed between these residues may be crucial for cooperative gating of the NMDA receptor by glutamate and glycine.

2. MATERIALS AND METHODS

2.1. *In vitro mutagenesis*

Oligonucleotide-directed mutagenesis was performed with single-stranded DNA of clone pN60 (NR1 cDNA; see [8]), or of a *KpnI*/*SacI* fragment (bp 1374–2327) thereof, using commercial kits (in vitro Mutagenesis System II, Amersham, and Muta-Gene Phagemid in vitro

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Mutagenesis Kit, BioRad). The tripletts encoding cysteine residues 4, 61, 290, 402, 418, 436, 437, 441, 726 and 780 of the mature NR1 subunit were substituted by alanine or other amino acid codons, respectively (see Fig. 1). All mutations were verified by dideoxy sequencing [18]. Mutant designations use the IUPAC single-letter amino acid code, with the letter preceding the position number referring to the wild-type residue, and the letter after the number corresponding to the amino acid replacing the former.

2.2. cRNA synthesis and oocyte expression

Linearized plasmid DNAs were used for the in vitro synthesis of cRNA (mCAP™mRNA Capping Kit, Stratagene). cRNAs of the rat NR1 [8] and the mouse NR2B [12] subunits were synthesized using T7 or T3 RNA polymerases, respectively. cRNA concentrations were determined by both measuring the optical density at 260 nm and comparing Methylene blue staining intensities after gel electrophoresis. For oocyte injection, the concentration of the cRNA samples was adjusted to 200–500 ng/μl. For co-expression experiments, appropriately diluted aliquots of the NR1 and NR2B cRNAs were mixed before injection to achieve an RNA ratio of 1:1. Microinjection of about 50 nl cRNA into *Xenopus laevis* oocytes and voltage clamp recording of agonist responses in Mg^{2+}-free frog Ringer's solution at a holding potential of −70 mV were performed as described [19].

3. RESULTS AND DISCUSSION

To investigate the role of disulfide bridges in agonist binding to the NMDA receptor, we used site-directed mutagenesis to replace the cysteine residues of the NR1 subunit (Fig. 1) by alanine or, in a few cases, other amino acid residues. The mutant proteins were then co-expressed with the NR2B subunit using the *Xenopus* oocyte system, and responses to glutamate and the co-agonist glycine were measured using two-electrode voltage-clamp recording in the absence of Mg^{2+} ions. Peak inward currents (I_{max}) obtained with saturating concentrations of L-glutamate in the presence of 10 μM glycine were in the range of 1000–3600 nA for the hetero-oligomeric wild-type and most of the mutant receptors (Table I). However, some cysteine substitutions resulted in significantly reduced ion fluxes: C402A, 40 ± 14 nA; C418A, 160 ± 100 nA; C418F, 25 ± 7 nA; and C418S, 53 ± 24 nA. Three mutants, $NR1^{C418L}$, $NR1^{C436D}$ and $NR1^{C437R}$, were non-functional (Table I). In conclusion, replacement of the cysteines at positions 402 and 418 of the NR1 subunit caused a strong decrease in NMDA receptor current.

To reveal whether this decrease could be attributed to changes in ligand binding, dose–response relations of the mutant NMDA receptors were examined for L-glutamate in the presence of 10 μM glycine and for glycine in the presence of 10 μM L-glutamate. Under these conditions, the agonist concentrations eliciting a half-maximal response (EC_{50}) varied from 0.7 to 3.0 μM for L-glutamate, and from 0.25 to 0.73 μM for glycine, respectively, with all mutants tested (Table I). These values are similar to those of the wild-type NR1/NR2B hetero-oligomeric channel, indicating that the low current responses of the cysteine 402 and cysteine 418 substitutions were not caused by a reduction of agonist binding affinities.

All hetero-oligomeric wild-type and mutant channels

Table I

Functional and pharmacological properties of NR1 cysteine mutants

	EC_{50} (μM)		I_{max} (μA)	Potentiation	IC_{50} (μM)
NR1 mutant	L-Glutamate	Glycine		(-fold)	7-Chlorokynurate
Wild-type	1.5 ± 0.5	0.52 ± 0.3	3.6 ± 1.0	62.0 ± 30.0	0.6 ± 0.3
C004A	2.8 ± 0.9	0.43 ± 0.3	2.3 ± 1.1	33.6 ± 25.0	0.9 ± 0.5
C061A	1.1 ± 0.5	0.25 ± 0.2	1.0 ± 0.25	36.2 ± 19.0	0.5 ± 0.2
C290A	1.5 ± 0.3	0.35 ± 0.1	2.2 ± 0.7	65.5 ± 21.0	0.6 ± 0.3
C402A	3.0 ± 1.2	0.47 ± 0.4	0.04 ± 0.014	3.5 ± 0.5	2.0 ± 0.3
C418A	2.8 ± 0.9	0.68 ± 0.3	0.16 ± 0.1	4.5 ± 4.1	0.8 ± 0.4
C418L	–	–	NF	–	–
C418F	ND	ND	0.025 ± 0.007	4.0 ± 0.9	NT
C418S	3.0 ± 0.8	0.72 ± 0.2	0.053 ± 0.024	5.7 ± 4.0	NT
C436A	1.3 ± 0.9	0.48 ± 0.2	1.2 ± 0.5	NT	0.5 ± 0.2
C436D	–	–	NF	–	–
C436S	1.7 ± 0.5	0.73 ± 0.3	2.7 ± 1.2	54.8 ± 26.0	0.8 ± 0.1
C437A	1.0 ± 0.6	0.70 ± 0.4	3.2 ± 1.0	NT	1.0 ± 0.8
C437R	–	–	NF	–	–
C437S	3.0 ± 1.2	0.25 ± 0.1	1.6 ± 0.1	55.3 ± 25.0	NT
C441A	3.0 ± 0.5	0.45 ± 0.1	0.95 ± 0.5	26.8 ± 13.0	0.5 ± 0.1
C726A	0.7 ± 0.3	0.25 ± 0.1	3.3 ± 1.1	15.2 ± 4.3	0.9 ± 0.5
C780A	0.7 ± 0.2	0.41 ± 0.3	3.1 ± 0.9	17.3 ± 3.8	1.4 ± 1.0

NR1 wild-type and mutant cRNAs were coinjected into *Xenopus* oocytes, and the receptors generated analyzed by voltage clamp recording as detailed under Material and Methods. EC_{50} values ± S.D. were calculated from dose-response curves obtained from 3–9 oocytes, each. IC_{50} values were measured at the EC_{50} value of glycine in the presence of 10 μM L-glutamate on 2–4 oocytes. Non-functional mutants were tested in ≥ 10 oocytes. I_{max} values were determined in the presence of saturating concentrations of L-glutamate and glycine, respectively. Potentiation of the responses evoked by single application of 10 μM L-glutamate and 10 μM glycine indicates the relative increase in total current seen upon coapplication of both agonists. ND – not determined because of low channel activities; NF – non-functional; NT – not tested.

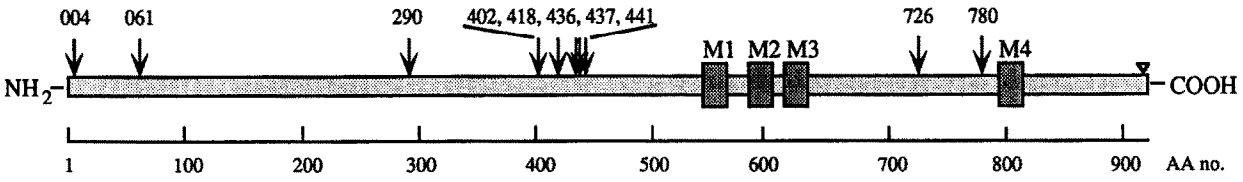

Fig. 1. Schematic representation of the NR1 subunit. Predicted membrane spanning regions (M1 to M4) are symbolized by boxes. The cysteines mutated in this study are indicated by arrows; numbers correspond to amino acid positions in the mature protein. A non-mutated cysteine in the very C-terminal region of the protein is indicated by a triangle.

displayed small current responses when challenged with 10 μM L-glutamate or glycine alone (Fig. 2A, and data not shown). With wild-type NR1/NR2B channels, the responses evoked by single agonist application were strongly (ca. 60-fold) enhanced upon co-application of both agonists (Fig. 2A, B). Similar results were obtained for the cysteine substitutions at positions 4, 61, 290, 436, 437 and 441 of the NR1 subunit (Table I). However, channels generated from the $NR1^{C402A}$ and $NR1^{C418A}$ mutant proteins showed only small increases in total current upon superfusion of both glutamate and glycine (Fig. 2 and Table I). Thus, cysteines 402 and 418 appear to be crucial for the cooperative gating of the NMDA receptor by glutamate and glycine. Interestingly, the $NR1^{C726A}$ and $NR1^{C780A}$ mutants also showed a significantly reduced glycine potentiation upon co-expression (Table I). This may indicate that the corresponding positions between the third and fourth transmembrane segment are localized on extracellular domains of the NMDA receptor. Recent models of glutamate receptor subunit topology are consistent with this interpretation [20].

Kynurenic acid and its derivatives are potent competitive antagonists of the glycine modulatory site of the NMDA receptor [21]. We therefore tested whether 7-chlorokynurenic acid inhibits mutant glutamate responses in the presence of glycine. For all NR1 cysteine substitutions producing a response, the concentrations of 7-chlorokynurenic acid causing half-maximal inhibition (IC_{50}) ranged between 0.5 to 2.0 μM, i.e. close to the IC_{50} value of the hetero-oligomeric wild-type receptor (Table I). This supports the view that the C402A and C418A mutations do not affect the properties of the glycine binding site, but disrupt glycine–glutamate interaction.

What might be the role of these cysteines in the cooperative gating of the NMDA receptor? In our view, the simplest explanation is that cysteines 402 and 418 form a disulfide bridge essential for conformational transitions induced upon agonist binding. Interestingly, a disulfide-bonded loop of similar size, e.g. covering 15 amino acid residues, is found in the N-terminal extracellular domain of other neurotransmitter-gated ion channel proteins, e.g. the subunits of the excitatory nicotinic

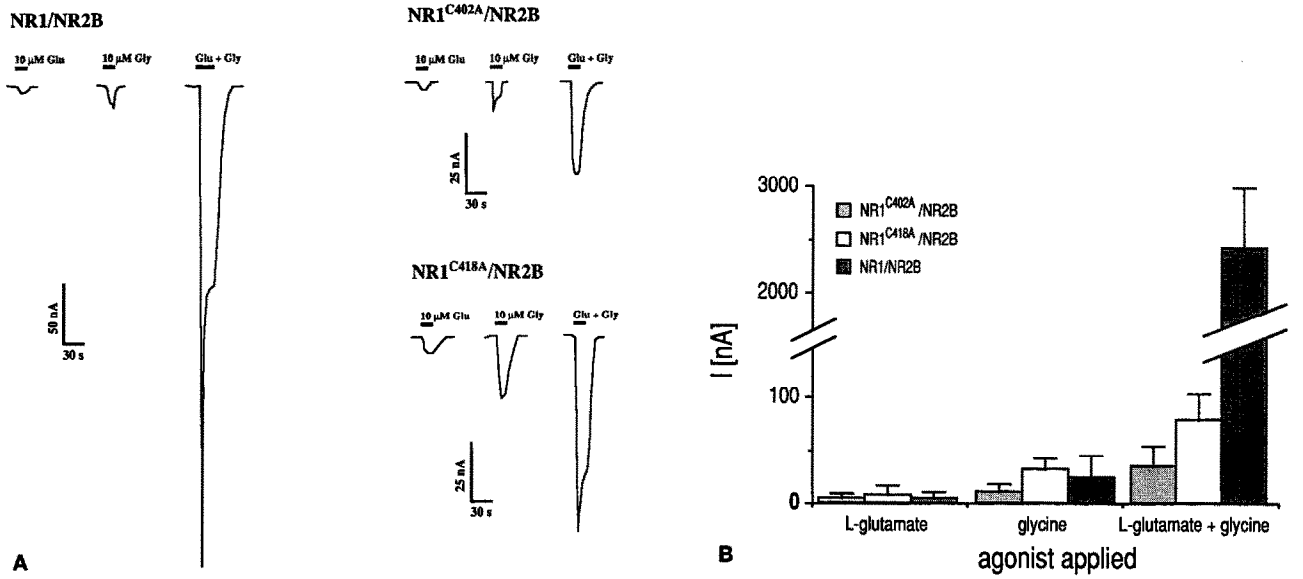

Fig. 2. Agonist responses of hetero-oligomeric NMDA receptors generated by functional co-expression of wild-type and mutant NR1 subunits with the NR2B polypeptide. *Xenopus* oocytes were injected with the cRNAs, and agonist responses were recorded as described in Section 2. (A) Inward current responses to 10 μM L-glutamate, 10 μM glycine and 10 μM L-glutamate plus 10 μM glycine of channels containing the wild-type NR1, or the mutant $NR1^{C402A}$ and $NR1^{C418A}$, subunits. The duration of agonist application is indicated by bars. (B) Mean agonist responses of the hetero-oligomeric NR1/NR2B and the mutant $NR1^{C402A}$/NR2B and $NR1^{C418A}$/NR2B receptors. Note that the large potentiation of the glutamate current by glycine seen with the wild-type receptor is largely abolished upon cysteine substitution. The data represent the mean ± S.D of 3–9 oocytes.

acetylcholine and serotonine (type 3) receptors and of the inhibitory $GABA_A$ and glycine receptor proteins [22,23]. For the nicotinic acetylcholine and glycine receptors, substitution of the flanking cysteine residues has been shown to strongly reduce the expression of functional channels [24–26], although this conserved disulfide loop region is known not to participate in agonist binding [25,27,28]. We therefore speculate that such disulfide bridges in the extracellular domain of ion channel forming receptors may provide folding patterns, which allow for rapid conformational changes elicited by ligand binding.

Previous electrophysiological work has shown that the native NMDA receptor has a redox modulatory site thought to consist of vicinal thiol groups, whose reduction increases current flux through the receptor channel without affecting glutamate–glycine interaction [29,30]. In the nicotinic acetylcholine receptor, two neighbouring cysteine residues preceding the first transmembrane segment have been found to be in close proximity to bound acetylcholine [31]. A similar pair of cysteines exists in the NR1 subunit at positions 436 and 437. In our hands, substitution of these residues by alanine or serine had no significant effect on agonist affinities and glycine potentiation (Table I). However, the incorporation of charged residues at these positions in mutants $NR1^{C436D}$ and $NR1^{C437R}$ rendered the channel non-functional. We interpret this as evidence for a structural role of these residues rather than their participation in ligand binding. Recent mutagenesis experiments from our laboratory show that side chains other than cysteines are important for agonist binding to the NMDA receptor (A. Kuryatov, B. Laube, H. Betz and J. Kuhse, in preparation).

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