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Subunit-specific block of cloned NMDA receptors by argiotoxin$_{636}$

Martin Raditsch[a], J. Peter Ruppersberg[a], Thomas Kuner[c], Willy Günther[a], Ralf Schoepfer[c], Peter H. Seeburg[c], Werner Jahn[b] and Veit Witzemann[a]

Abteilung [a]Zellphysiologie and [b]Biophysik, Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, W-6900 Heidelberg, Germany and *[c]Center for Molecular Biology, University of Heidelberg, Im Neuenheimer Feld 282, Heidelberg, Germany*

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Cloned NMDA receptor channels of the NR1-NR2A, NR1-NR2B and NR1-NR2C type show differences in argiotoxin$_{636}$ block. Mutations of an asparagine residue located at a homologous position in the TM2 region of all NMDA receptor subunits, which corresponds to the Q/R site of the AMPA receptors, alters the argiotoxin$_{636}$-induced block. The results suggest that the toxin interacts at this amino acid position with the putative pore forming TM2 region of the NMDA receptor subunits. Sequence differences in the TM2 segment of NR2A and NR2C subunits are not responsible for the subtype-specific sensitivity to argiotoxin$_{636}$ as revealed by site-directed mutagenesis.

NMDA receptor; Recombinant; Argiotoxin; Site-directed mutagenesis

1. INTRODUCTION

Several low molecular weight spider and wasp toxins, such as argiotoxins [1], philanthotoxin [2], joro spider toxin [3] and α-agatoxins [4], paralyse insects by blocking glutamatergic neuromuscular transmission. These arylamine spider toxins also interact with mammalian glutamate receptors of the brain [5–7]. The recent cloning and functional expression of various mammalian glutamate receptor subtypes [8] provided an opportunity to correlate receptor-specific toxin sensitivities with individual subunits. We analysed the blockade of recombinant AMPA receptors by argiotoxin$_{636}$ (ATX) and demonstrated the expression of heteromeric receptor complexes where ATX insensitivity was determined by the presence of GluR-B subunits [9]. Channels with largely reduced ATX sensitivity also had a low Ca^{2+} permeability which depended on the presence of a positively charged arginine residue in the second putative transmembrane segment (TM2) of the GluR-B subunit [10,11]. GluR-A, GluR-C and GluR-D contain, in contrast to GluR-B, an uncharged glutamine residue at the homologous position in TM2. These homomeric and heteromeric channels were effectively blocked by ATX [9]. The amino acid residue at the homologous position in the TM2 sequence of all cloned NMDA receptor subunits is asparagine [12], suggesting that various NMDA receptor subtypes may have similar sensitivities towards ATX. We therefore compared the interaction between chemically synthesized ATX and heteromeric NR1-NR2A, NR1-NR2B and NR1-NR2C receptors [12,13] in *Xenopus* oocytes. The observed differences in ATX sensitivity of these three receptor types and the role of the putative pore forming region, TM2, were investigated by site-directed mutagenesis.

Correspondence address: V. Witzemann, Abteilung Zellphysiologie, Max-Planck-Institut für medizinische Forschung, Jahnstraße 29, W-6900 Heidelberg, Germany. Fax: (49) (6221) 486 459.

2. MATERIALS AND METHODS

Argiotoxin$_{636}$ (ATX) was synthesized as described previously [6,14]. Glutamate and glycine were purchased from Sigma. Complementary DNAs encoding the NMDA receptor subunits and mutated subunits were subcloned into pSP64T [15] derived vectors [16]. The genuine 5′ and 3′ untranslated regions were mostly removed. Plasmids were linearized 3′ of the poly(A) stretch. NMDA receptor subunit-specific cRNAs were synthesized in vitro using SP6-polymerase [15]. *Xenopus laevis* oocytes were injected with 50 nl of cRNA (approx. 1 mg/ml) and incubated at 19°C in modified Barth's medium containing penicillin and streptomycin. 4 h after cRNA injection, oocytes were treated with collagenase type I (Sigma) (1 mg/ml) to remove the follicle cell layer. Agonist-activated currents were recorded 24–120 h after injection at room temperature and at −70 mV using a two-microelectrode voltage clamp. Oocytes were perfused continuously with modified frog Ringer's solution (Ba^{2+}FR) containing 1.8 mM $BaCl_2$, 115 mM NaCl, 2.5 mM KCl and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) adjusted to pH 7.2. Ba^{2+} was used in order to reduce Ca^{2+}-evoked chloride currents in oocytes [17]. Both agonists, glutamate (100 μM) and glycine (10 μM), and the antagonist, ATX, were dissolved in Ba^{2+}FR. Current and voltage electrodes were filled with 3 M KCl and had resistances between 0.5 and 3 MΩ. Current responses were recorded on a Graphtec chart recorder. The inhibition of NMDA receptor-mediated currents were measured by switching from Ba^{2+}FR to agonist-containing Ba^{2+}FR to activate the NMDA receptor channels. Inhibition was induced by perfusing with Ba^{2+}FR containing agonists and ATX at varying concentrations. At least five independent experiments were performed for each ATX concentration and reduction of current in response to ATX was determined 5 min after addition of the toxin. The percentage of residual current in the presence of ATX was plotted vs. ATX concentration. The curves were fitted by the equation $I = I_o/(1 + ([ATX]/IC_{50})^n)$ to determine the concentration of half-maximal block (IC_{50}), where I is the amplitude of

evoked current using a defined ATX concentration ([ATX]) and I_o is the response in the absence of ATX. Hill coefficients (n) were slightly below unity in all measurements.

3. RESULTS

Fig. 1A shows that ATX acts differentially on NMDA receptor channels composed of NR1-NR2A or NR1-NR2C subunits. NR1-NR2B receptors respond to ATX with a similar sensitivity as NR1-NR2A receptors (Table I). Application of 100 nM ATX in presence of 100 μM glutamate and 10 μM glycine blocked > 90% of the NR1-NR2A channels but exerted only a weak effect on the current response mediated by NR1-NR2C receptors. Concentration-dependent inhibition curves were constructed from measurements, as shown in Fig. 1A. Varying the ATX concentrations from 0.01 to 100,000 nM revealed IC_{50} values of 9 nM for NR1-NR2A, 4.5 nM for NR1-NR2B and 460 nM for NR1-NR2C channels (Fig. 1B and Table I). This difference in sensitivity of NMDA receptor subtypes cannot be explained by a charge difference in the TM2 region at the recently described Q/R site, since all NMDA receptor subunits carry an asparagine at the homologous position (Fig. 2).

In order to test the functional role of this site in NMDA receptor subunits regarding the action of ATX, we changed the native asparagine to either glutamine or arginine. The glutamine-containing NR2A and NR2C mutants co-expressed with NR1 subunits yielded heteromeric channels which displayed a significant increase in ATX sensitivity compared to wild-type channels. The IC_{50} = 1 nM for NR1-NR2A (N595Q) was increased 9-fold and the IC_{50} = 22 nM for NR1-NR2C (N593Q) 20-fold (Fig. 3 and Table I). An even higher increase was observed when both NR1 and NR2A subunits carried the asparagine-to-glutamine mutation and the

Table I

IC_{50} values of wild-type and mutated NMDA receptor channels

NMDA receptor channel	IC_{50} (nM)
NR1-NR2A	9
NR1 (N598Q)-NR2A	10
NR1-NR2A (N595Q)	1
NR1 (N598Q)-NR2A (N595Q)	0.2
NR1-NR2A (N595R)	n.d *
NR1-NR2B	4.5
NR1-NR2C	460
NR1-NR2C (N593Q)	22
NR1-NR2C (SVA-AIG)	340
NR1-NR2C (E599Q)	440

ATX concentration-dependent block of NMDA receptor channels was determined as described in Fig. 1. Each value corresponds to the values from 5–10 experiments.
*At 10,000 nM ATX 25% of the agonist-induced current was blocked

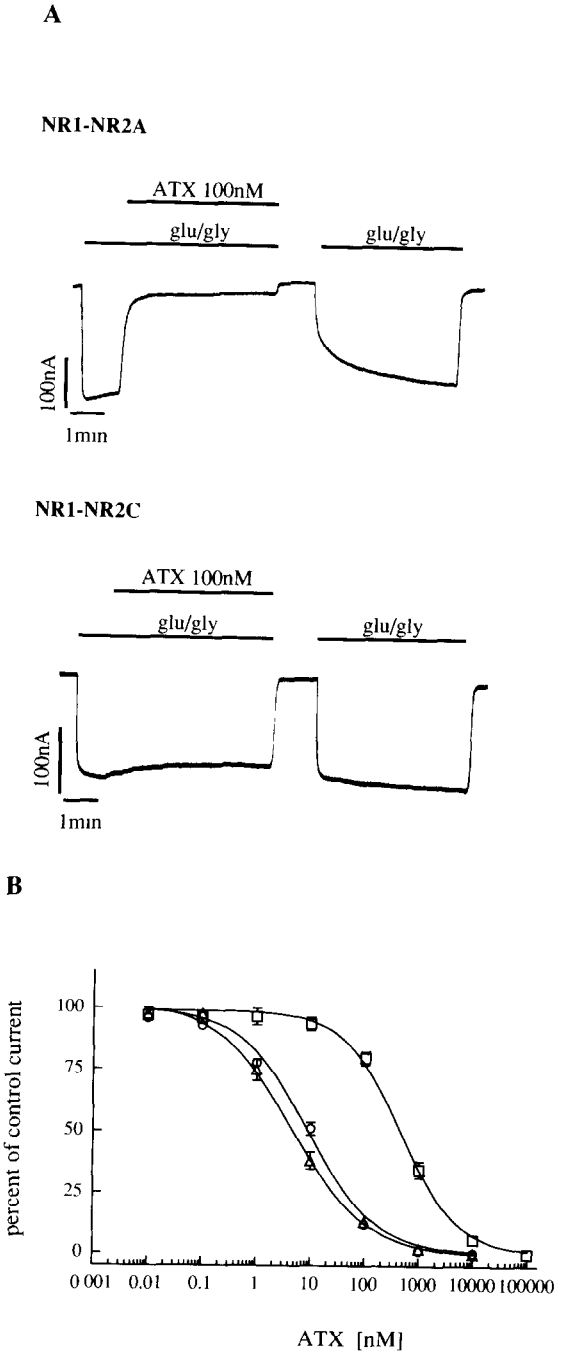

Fig. 1. Differential ATX sensitivity of heteromeric NMDA receptor channels. (A) Effect of 100 nM ATX on the inward current mediated by NR1-NR2A and NR1-NR2C channels in response to 100 μM L-glutamate and 10 μM glycine (glu/gly) at −70 mV membrane potential. The slow activation of NR1-NR2A upon ATX block and wash out reflects on the slow time-course of recovery. (B) ATX concentration-dependent inhibition of currents mediated by NR1-NR2A, NR1-NR2B and NR1-NR2C NMDA receptor channels. Current responses expressed in percent of control were measured as in A, and normalized to the current in the absence of ATX. Error bars indicate S.E.M. of 5–10 independent experiments. The IC_{50} values of ATX block on NR1-NR2A (○) and NR1-NR2B (△) are similar; in contrast the ATX sensitivity of NR1-NR2A and NR1-NR2C (□) differs by about 50-fold. IC_{50} values are given in Table I.

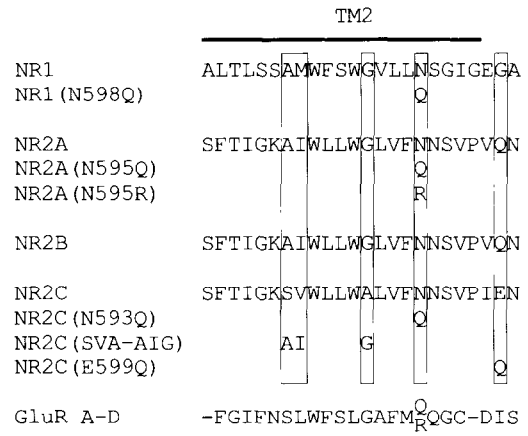

Fig. 2. Wild-type and mutated sequences of the TM2 region of the NMDA receptor subunits. Alignment of wild-type sequences of the putative TM2 segment of NR1, NR2A, NR2B, NR2C and AMPA receptor subunits (GluR A–D) [8,16,18]. Mutated amino acid residues analysed here are boxed. The valine (V600) of NR2A flanking the putative TM2 region at the C-terminal end is replaced by an isoleucine (I598) in NR2C. A possible contribution of this amino acid to ATX block has not been analysed.

channels were blocked with an IC_{50} value of 0.2 nM (Table I).

A dramatic reduction in the ATX sensitivity was observed when the asparagine of the NR2A subunit was replaced by the positively charged arginine. NR1-NR2A (N595R) had a greatly reduced sensitivity for ATX, and at 10,000 nM ATX concentrations the agonist-induced current responses were blocked only by 25% (Table I). Thus, the single amino acid residue in the TM2 region of the NMDA receptor subunits determines the ATX sensitivity of the NMDA receptors. The results, however, do not explain the pronounced differences in sensitivity between NR1-NR2A or NR1-NR2B and NR1-NR2C receptor subtypes.

To investigate whether these differences were determined by other single amino acids residing in the TM2 region of the NMDA receptor subunits we replaced in NR2C all residues which differed from the NR2A amino acid sequence (exchanges are shown in Fig. 2). The resulting NR1-NR2C (SVA-AIG) channels, containing the same amino acid sequences in their TM2 segments as the NR1-NR2A channels, exhibited a similar ATX sensitivity as the wild-type NR1-NR2C channels (Table I). Thus, subunit-specific differences cannot be traced to TM2.

The NR2A- and NR2C-specific sequences differ in the region C-terminal to TM2, in close proximity to the critical asparagine residue. The uncharged glutamine (Q602) of NR2A corresponds to a negatively charged glutamate (E599) in NR2C. The expression of mutant channels having the glutamate in the NR2C subunit replaced by glutamine residue, NR1-NR2C (E599Q), had no effect on ATX sensitivity. Hence, this sequence difference is not responsible for the subtype-specific ATX blockade.

4. DISCUSSION

The asparagine residue in the putative pore-forming TM2 region of NMDA receptor subunits corresponds to the Q/R site of the AMPA receptor subunits. This site determines ATX sensitivity [9] and Ca^{2+} permeability [10,11]. Using site-directed mutagenesis we can show that this position is critical for sensitivity of the NMDA receptor channels to ATX. An increase in ATX sensitivity was observed when glutamine replaced the native asparagine, while arginine caused a dramatic decrease. Thus, as already observed for the AMPA receptor channels, a single amino acid position in the TM2 region determines ATX sensitivity. A positively charged arginine residue renders both channels insensitive to ATX block. Blockade is prevented by repulsive forces since ATX itself carries a positively charged arginine residue at the end of a polyamine tail [1]. Interestingly, however, glutamine and asparagine have different effects in AMPA and NMDA receptor channels, respectively. NMDA receptor subunits become more sensitive to ATX when replacing the native asparagine by glutamine. In contrast, higher sensitivity was found for the AMPA receptors containing an asparagine instead of a glutamine residue at the Q/R site in TM2 [9]. These differences may reflect the distinct topology of the two ion channels.

Since at this channel position there is no difference between the native NR2A, NR2B and NR2C subunits, the large difference in ATX sensitivity between NR1-NR2A and NR1-NR2C channels must be conferred by other structural determinants. The low ATX sensitivity of NR2C could not be increased by converting the TM2 sequence to that of the NR2A subunit. Neither did the

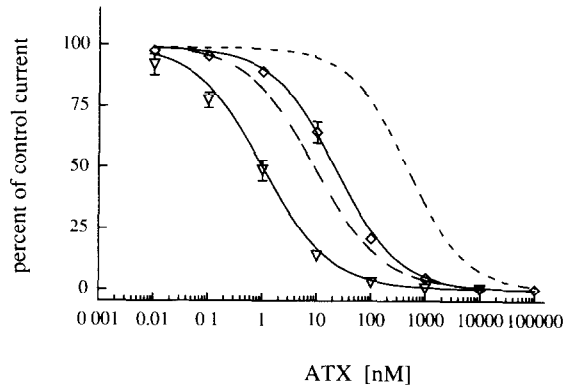

Fig. 3. Asparagine to glutamine mutations in the TM2 region increase ATX sensitivity of NR1-NR2A and NR1-NR2C channels. ATX sensitivity of mutated NMDA receptor channels NR1-NR2A (N595Q) (▽) and NR1-NR2C (N593Q) (◇) in comparison to the dose–response curves of the wild-type channels NR1-NR2A (—) and NR1-NR2C (----). Concentration-dependence of ATX block was determined as in Fig. 1.

charge difference of an amino acid residue C-terminal to the putative TM2 region, NR2C (E599) explain the 50-fold higher sensitivity of NR2A channels towards ATX.

Thus the subunit-specific differences in ATX block between NR1-NR2A and NR1-NR2C channels are not due to sequence differences in the putative pore-forming segment, TM2. The 50-fold difference in ATX sensitivity of the two NMDA receptor subunit types needs further investigation by site-directed mutagenesis. This experimental approach should reveal which parts of the channel other than the TM2 region are involved in the interaction with arylamine toxins. Furthermore, the large subunit-specific difference in ATX sensitivity described here may serve as a tool to elucidate the subunit compositions of native NMDA receptors, or even help to determine subunit stoichiometries of synaptic NMDA receptors channels.

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