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Anthraquinone polyamines: novel channel blockers of *N*-methyl-D-aspartate receptors

Review Article

K. Kashiwagi[1], **K. Williams**[2], and **K. Igarashi**[3]

[1] Faculty of Pharmacy, Chiba Institute of Science, Choshi, Japan
[2] Department of Physiology and Pharmacology, State University of New York Downstate Medical Center, Brooklyn, New York, USA
[3] Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan

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Summary. Polyamines, in particular spermine, as well as some natural and synthetic polyamine derivatives have been found to be blockers of *N*-methyl-D-aspartate receptors. We developed novel, polyamine-based channel blockers to analyze the structure of NMDA receptors. Anthraquinone polyamines block NMDA receptors with some selectivity compared to other glutamate receptors. Results using mutant NR1 and NR2 subunits identified amino acid residues that influence blockade by anthraquinone polyamines. The head group (anthraquinone) may be positioned at the selectivity filter/narrowest constriction of the channel and the polyamine tail penetrates this constriction into the inner vestibule below the level of the selectivity filter. The results are consistent with other work showing that NR1 (Asn616) and NR2B (Asn616), but not NR2B (Asn615), make the narrowest constriction of NMDA channel, and that the M3 segments from the two subunits, which form the outer vestibule, are likely staggered relative to each other in the vertical axis of the channel.

Keywords: Polyamine derivatives – Glutamate receptor – NMDA receptor – Channel blocker

Abbreviations: AMPA, α-aminio-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AQP, anthraquinone polyamine; AQ33, N^1-(anthraquinone-2-carbonyl)norspermidine; AQ33b, 4-(anthraquinone-2-carbonyl)-4-azaheptane-1,7-diamine; AQ34, N^1-(anthraquinone-2-carbonyl)spermidine; AQ343, N^1-(anthraquinone-2-carbonyl)spermine; AQ444, N^1-(anthraquinone-2-carbonyl)homospermine; MK801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; TB34, N^1,N^4,N^8-tribenzylspermidine

Introduction

N-Methyl-D-aspartate (NMDA) receptors are a subtype of ligand-gated ionotropic glutamate receptors including subtypes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Dingledine et al., 1999). Although they share common structural features, NMDA receptors have several characteristics different from the other two subtypes of glutamate receptors; 1) Not only glutamate but also glycine are necessary for activation of NMDA receptors; 2) NMDA receptors show high permeability to Ca^{2+}; and 3) NMDA receptor activity is blocked by Mg^{2+} at the resting membrane potential and the block is relieved by depolarization (Dingledine et al., 1999). Because of these characteristics, NMDA receptors play pivotal roles for synaptic plasticity, bring about neuronal cell death by their overactivation, and may become potential targets for neuroprotective agents and anticonvulsants (Choi, 1988; Rogawski, 1992). The NMDA receptors are probably tetramers composed of two molecules each of NR1 and NR2 subunits. There is one gene for NR1 subunits and four genes for NR2 subunits, NR2A-NR2D (Dingledine et al., 1999). Most NMDA receptors in the adult central nervous system contain combinations of NR1 and NR2, with NR2A and NR2B predominating in forebrain areas such as the cerebral cortex (Dingledine et al., 1999; Hollmann and Heinemann, 1994; Watanabe et al., 1992). Each subunit consists of a extracellular N-terminal regulatory (R) domain, agonist binding (S) domain, channel forming domain and intracellular domain (Fig. 1). There are four membrane (M1–M4) region in the channel forming domain, and the second membrane segment (M2) is thought to form a re-entrant loop and contribute to the narrowest constriction of the channel

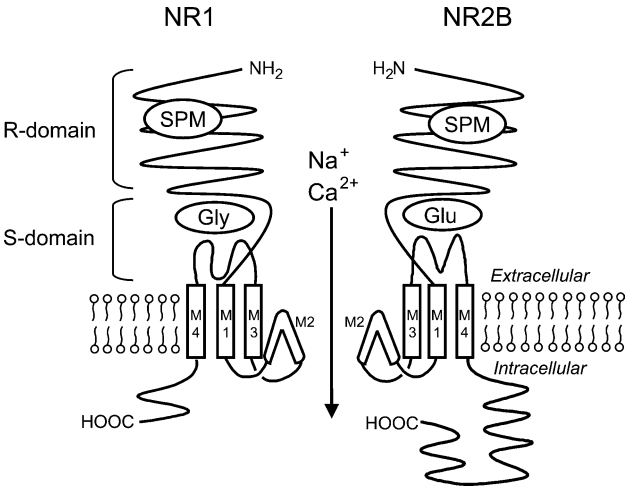

Fig. 1. Structure of NMDA receptors (NR1/NR2B). NMDA receptors consist of regulatory (R) domain, agonist binding (S) domain, three membrane-spanning domains (M1, M3 and M4) and a re-entrant loop (M2) that forms part of the channel pore. The M3 domain is most strongly involved in the formation of channel among three membrane-spanning domains

(Fig. 1). The M2 loop region in NR1 and NR2 subunits is a critical determinant of divalent cation permeability and Mg^{2+} blockade. In particular, asparagine residues in this region form part of the Mg^{2+} binding site and contribute to the selectivity filter of the channel (Dingledine et al., 1999).

Polyamines, especially spermine has multiple effects on NMDA receptors, including stimulation at the depolarized membrane potential and a weak voltage-dependent inhibition due to open-channel block (Benveniste and Mayer, 1993; Williams, 1997). A number of synthetic polyamine derivatives and natural, polyamine-derived spider toxins have also been shown to block the NMDA channel, and such compounds are useful as tools to study the structure and function of glutamate receptors (Igarashi and Williams, 1995; Igarashi et al., 1997; Jackson and Usherwood, 1988). The asparagine residues located in the M2 loop region of NMDA receptor have also been found to influence block by organic channel blockers such as MK-801, memantine, and polyamine derivatives such as N^1,N^4,N^8-tribenzylspermidine (TB34) (Kashiwagi et al., 2002; Sakurada et al., 1993). Amino acid residues in the M1, M3, and M4 regions of NR1 and NR2 subunits have also been found to affect block by polyamine derivatives (Kashiwagi et al., 2002).

Recently, we found that anthraquinone polyamine (AQP) derivatives also act as preferential NMDA channel blockers. Using this new type of NMDA channel blockers, the structure of the channel was studied in more detail (Jin et al., 2007; Kashiwagi et al., 2004).

NMDA channel block activity of AQP derivatives

The effects of AQ polyamines were studied at recombinant NMDA receptors expressed in frog oocytes. A number of

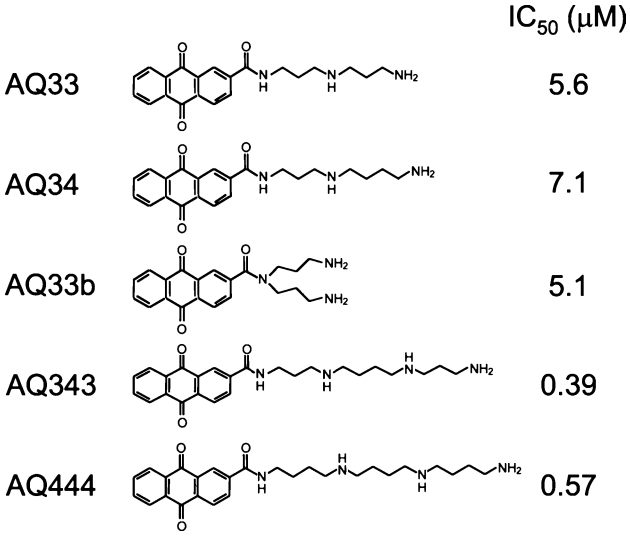

Fig. 2. Structures and channel block activity of AQP derivatives. IC_{50} values were determined from concentration-inhibition curves ($n = 4$) at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV

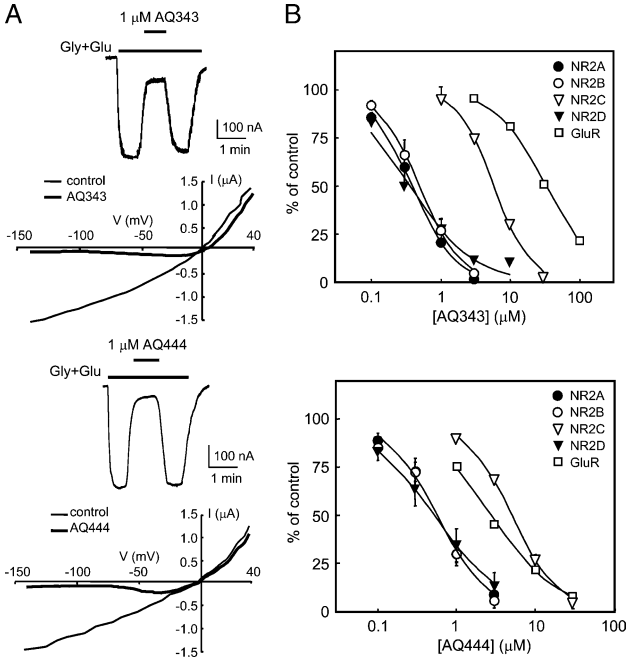

Fig. 3. Effects of AQ343 and AQ444 at NMDA and AMPA receptors. **A** Representative traces are shown to illustrate the effect of 1 μM of AQ343 and AQ444 on currents activated by Glu + Gly at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV. I–V curves for Glu + Gly-activated currents were measured by voltage ramps in the absence and presence of AQ343 and AQ444. **B** Concentration-inhibition curves were determined at NR1/NR2 receptors containing NR2A, NR2B, NR2C and NR2D subunits and at AMPA receptors expressed from GluR1 in oocytes voltage-clamped at −70 mV

AQ derivatives, having different polyamine tails, were studied (Fig. 2). All the compounds showed voltage-dependent inhibition of recombinant NR1/NR2A receptors, consistent with a channel-blocking mechanism. At −70 mV, IC_{50} values ranged from 0.6 to 6 μM (Fig. 2), and potency appears to be related to the number of positive charges and the size of polyamine tail.

Since AQ343 and AQ444 were potent inhibitors, we used these compounds to study the properties of channel block by AQ polyamines in detail. As shown in Fig. 3A, the inhibition was reversible and strongly voltage-dependent. The subunit selectivity of these compounds was also studied with recombinant NMDA receptors containing different NR2 subunits and at AMPA receptors expressed from GluR1. AQ343 and AQ444 were more potent at NR1/NR2A, NR1/NR2B and NR1/NR2D receptors than at NR1/NR2C and GluR1 receptors (Fig. 3B).

In addition to its inhibitory effects, spermine also potentiates responses at NR1/NR2B receptors through binding to an extracellular regulatory domain (Masuko et al., 1999; Williams, 1997). AQ343 and AQ444 did not potentiate responses to glutamate at NR1/NR2B receptors, suggesting that AQ343 and AQ444 only bind to the channel region, not to the polyamine site on the regulatory domain of NMDA receptors.

Identification of amino acid residues that influence block by AQ343 and AQ444

We carried out experiments to identify the amino acid residues in NR1 and NR2B that influence block by AQ343 and AQ444 using a series of NR1 and NR2B mutants (Fig. 4). In Fig. 4, mutations that reduce block by more than 15% compared to wild-type are highlighted by an open box, and mutations that increase block by AQ343 and AQ444 are highlighted by a black box. These residues were located in the outer vestibule and at the selectivity filter/narrowest constriction of the channel (Asn616 in NR1 and Asn615 and Asn616 in NR2B); see Fig. 5. We compared the effects on block by AQ polyamines with that of another polyamine derivative, TB34 (Kashiwagi et al., 2002). Eight mutations, at NR1 Phe558, Trp563, Asn616, Asn650, Ala652, Leu655, Asp669 and Thr807, reduced block by AQ343 and AQ444 and also by TB34

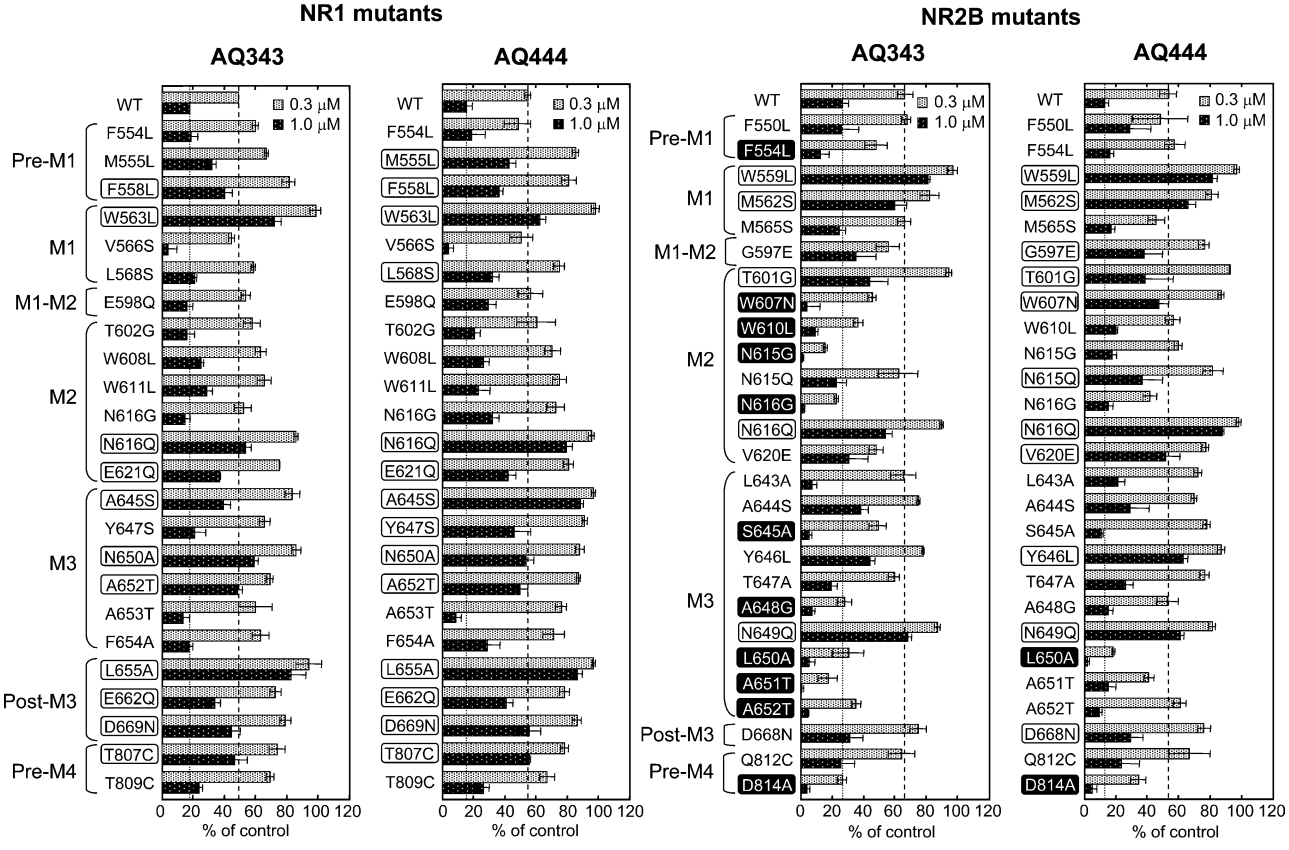

Fig. 4. Effects of AQ343 and AQ444 at receptors containing NR1 mutants or NR2B mutants. Mutations that reduced block by ≥15% compared to wild-type are highlighted by an open box. Mutations that enhanced block compared to wild-type are indicated by white letters in black boxes

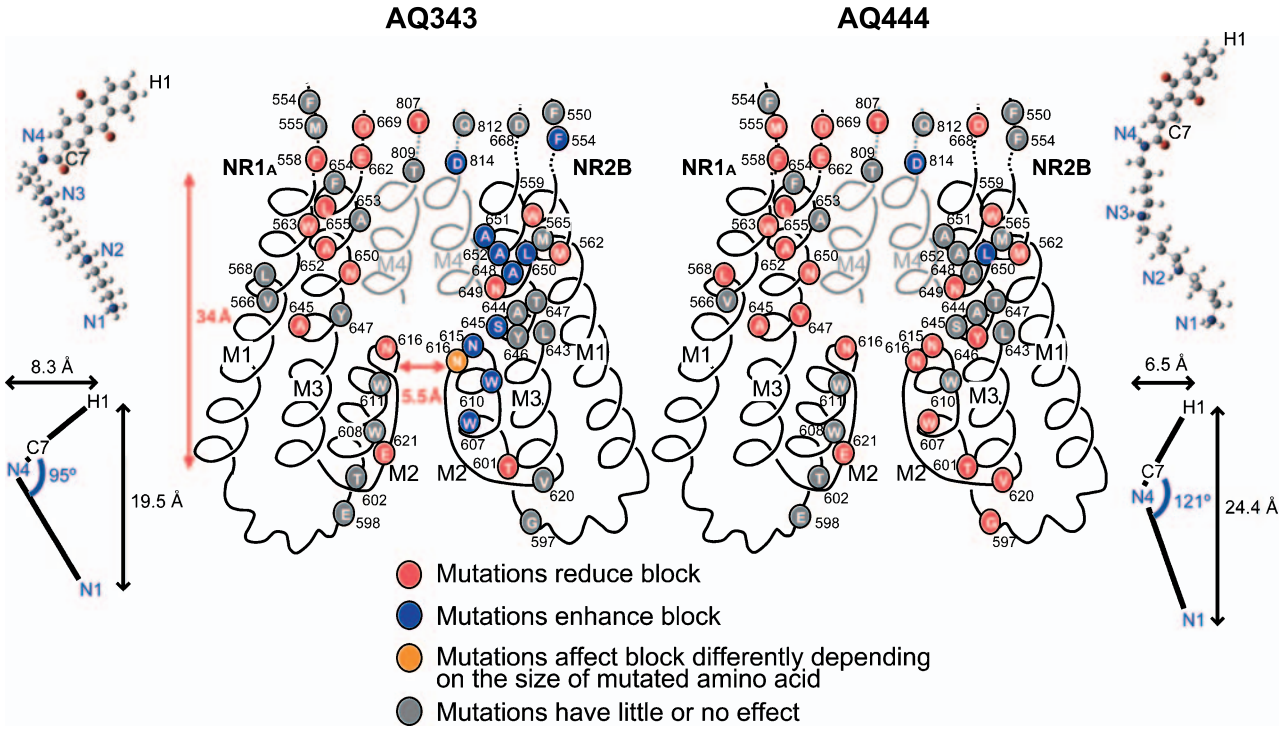

Fig. 5. Modeling residues that affect block by AQ343 and AQ444 in the pore and vestibule of the NMDA receptor, and preferred conformations of AQ343 and AQ444. The M1–M2–M3 region in depicted as a helix-pore-loop helix similar to the structure reported for the KcsA potassium channel from *Streptomyces lividans* (Doyle et al., 1998). The M2 region contains a helix followed by a random coil structure. Red circles indicate residues at which mutations reduce block by $\geq$15% compared to wild-type as shown in Fig. 4. Blue circles indicate residues at which mutations enhance block compared to wild-type. The yellow circle indicates residues at which mutations affect block differently depending on the size of mutated amino acid. Grey circles indicate residues at which mutations have little or no effect. Most stable conformations of AQ343 and AQ444 determined by molecular dinamics simulation were shown together with horizontal and vertical length

(Jin et al., 2007; Kashiwagi et al., 2002, 2004). This suggests that these residues are involved in the recognition of all of these compounds. With regard to residues in the inner vestibule below the level of the selectivity filter, Glu621 in NR1 influenced block by AQ343 and AQ444. This observation supports the idea that the polyamine tail passes through the selectivity filter and can interact with residues below that level whereas the AQ head group cannot easily permeate the narrow constriction.

The profiles measured with mutations in NR2B were different from those in NR1 (Figs. 4 and 5). Mutations at NR2B Trp559, Asn616 and Asn649 decreased the block by AQ343 and AQ444 and also by TB34 (Jin et al., 2007; Kashiwagi et al., 2002, 2004). Mutations at only a few residues in the outer vestibule in NR2B reduced block by AQ343 and AQ444 compared with residues in the corresponding region of NR1. On the other hand, mutations at several residues, especially to the smaller residues Ala or Gly, enhanced block by AQ343, but did not affect block by AQ444. This may reflect differences in the space occupied by these compounds (Fig. 5). The ''width'' of AQ343 (8.3 Å) was greater than that of AQ444 (6.5 Å). The differences between NR1 and NR2B, seen mainly in the extracellular M3 segment, are consistent with the idea that the M3 segments from the two subunits are staggered relative to each other in the vertical axis of the channel (Sobolevsky et al., 2002).

Mutations at Gly597, Thr601, Trp607, and Val620 in the inner vestibule of NR2B reduced block by AQ444, but only a mutation at Thr601 reduced block by AQ343. On the contrary, mutations at Trp607 and Trp610 of NR2B enhanced block by AQ343. This may be due to a difference of the structure of AQ343 and AQ444 in which AQ444 is straighter than AQ343 as well as having a longer polyamine tail (Fig. 5).

Effects of mutations at the selectivity filter

An Asn to Gln mutation at the critical asparagine in the M2 loop of NR1 (N616Q) reduced block by AQ343 and AQ444 (Fig. 4); however, substitution of Gly for Asn did not influence significantly the block by AQ343 and

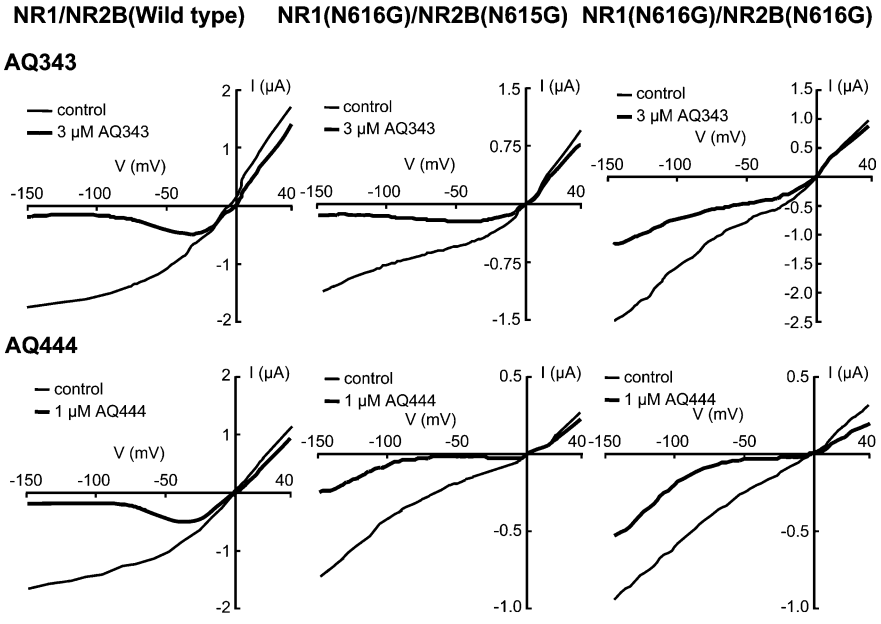

Fig. 6. Block and permeation of NMDA channels by AQ343 and AQ444. I–V curves were constructed by voltage ramps from −150 to +40 mV in oocytes expressing wild-type and mutant receptors. Responses to 10 μM glutamate (with 10 μM glycine) were measured in the absence and presence of the blocker

AQ444. There are two asparagine residues (Asn615 and Asn616) at a similar position in the M2 loop of NR2B, which also contribute to the selectivity filter and Mg^{2+} binding site (Dingledine et al., 1999). An N616G mutation in NR2B increased block by AQ343, and did not influence significantly the block by AQ444. An N616Q mutation at this position decreased block by AQ343 and AQ444. The results may be explained as follows: narrowing of the channel by substitution of Gln for Asn at N616 in NR1 or NR2B which decreased the block by these compounds, maybe due to the disturbance of penetration of the polyamine tail into the inner vestibule. Expanding of the channel (NR2B N616G) enhanced block by AQ343 preferentially. This may reflect occupancy of a wider space by AQ343 than by AQ444 (Fig. 5).

We carried out experiments to determine whether AQ343 and AQ444 could permeate wild-type and mutant NMDA channels (Fig. 6). To do this, we measured block and looked for relief of block at extreme negative membrane potentials (Chao et al., 1997). At wild-type receptors, block was almost complete from −100 to −150 mV (Fig. 6). However, by expanding the size of channel pore with NR1(N616G) and NR2B(N615G) or NR2B(N616G), AQ343 and, in particular, AQ444 showed significant permeation of the channel, characterized by a partial relief of block at very negative membrane potentials (Fig. 6).

Conclusions

We probed the interaction of AQ343 and AQ444 with the NMDA channel by using a large series of NR1 and NR2B mutants. We found that mutations in the M3 region in the outer vestibule of NR1 generally had greater effects on the blockers than mutations in the equivalent region of NR2B. The polyamine tail may pass through the narrowest constriction of the channel, and its interaction with the M2 loop and inner vestibule may be dependent on the angle of the head group and the polyamine tail. In this regard, AQ444, which has a longer polyamine tail than AQ343, was influenced by residues deeper in the inner vestibule. The data are consistent with the proposal that NR1 Asn616 and NR2B Asn616 form the narrowest constriction of the channel, with the NR1 and NR2 subunits arranged asymmetrically and that the M3 region in the outer vestibule of NR1 is strongly involved in the recognition of blockers (Dingledine et al., 1999; Kashiwagi et al., 2002, 2004; Wollmuth et al., 1996). However, some amino acid residues in M3 of NR2B also affected block by AQ343, but not AQ444. This may be due to the difference of space occupied by these compounds, because substitution of larger amino acid residues with smaller ones enhanced block by AQ343, but not by AQ444.

Among NMDA channel blockers, memantine has been used clinically in the treatment of Alzheimer's disease (Reisberg et al., 2003). Memantine is a readily reversible and selective channel blocker (Lipton, 2005) that may have better clinical utility and fewer side-effects than the very high affinity and slowly reversible blockers such as MK-801 (Huettner and Bean, 1988). The AQ polyamine derivatives have potencies similar to memantine, but better selectivity for NMDA receptors. AQ derivatives may be potentially useful lead compounds for the development

of therapeutically useful NMDA antagonists. Subtle molecular shape differences involving the angle between the polycyclic ring and the linear polyamine tail as well as the length of the polyamine tail itself are key parameters to be considered in the design of polyamine-derived NMDA receptor antagonists.

References

Benveniste M, Mayer ML (1993) Multiple effects of spermine on *N*-methyl-D-aspartic acid receptor responses of rat cultured hippocampal neurons. J Physiol 464: 131–163

Chao J, Seiler N, Renault J, Kashiwagi K, Masuko T, Igarashi K, Williams K (1997) N^1-Dansyl-spermine and N^1-(*n*-octanesulfonyl)-spermine, novel glutamate receptor antagonists: block and permeation of *N*-methyl-D-aspartate receptors. Mol Pharmacol 51: 861–871

Choi DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1: 623–634

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. Pharmacol Rev 51: 7–61

Doyle DA, Cabral JM, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science 280: 69–77

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17: 31–108

Huettner JE, Bean BP (1988) Block of *N*-methyl-D-aspartate-activated current by the anticonvalsant MK-801: selective binding to open channels. Proc Natl Acad Sci USA 85: 1307–1311

Igarashi K, Williams K (1995) Antagonist properties of polyamines and bis(ethyl)polyamines at *N*-methyl-D-aspartate receptors. J Pharmacol Exp Ther 272: 1101–1109

Igarashi K, Shirahata A, Pahk AJ, Kashiwagi K, Williams K (1997) Benzyl-polyamines: novel, potent *N*-methyl-D-aspartate receptor antagonists. J Pharmacol Exp Ther 283: 533–540

Jackson H, Usherwood PNR (1988) Spider toxins as tools for dissecting elements of excitatory amino acid transmission. Trends Neurosci 11: 278–283

Jin L, Sugiyama H, Takigawa M, Katagiri D, Tomitori H, Nishimura K, Kaur N, Phanstiel IV O, Kitajima M, Takayama H, Okawara T, Williams K, Kashiwagi K, Igarashi K (2007) Comparative studies of anthraquinone- and anthracene-tetraamines as blockers of *N*-methyl-D-aspartate receptors. J Pharmacol Exp Ther 320: 47–55

Kashiwagi K, Masuko T, Nguyen CD, Kuno T, Tanaka I, Igarashi K, Williams K (2002) Channel blockers acting at *N*-methyl-D-aspartate receptors: differential effects of mutations in the vestibule and ion channel pore. Mol Pharmacol 61: 533–545

Kashiwagi K, Tanaka I, Tamura M, Sugiyama H, Okawara T, Otsuka M, Sabado TN, Williams K, Igarashi K (2004) Anthraquinone polyamines: novel channel blockers to study *N*-methyl-D-aspartate receptors. J Pharmacol Exp Ther 309: 884–893

Lipton SA (2005) The molecular basis of memantine action in Alzheimer's disease and other neurologic disorders: low-affinity, uncompetitive antagonism. Curr Alzheimer Res 2: 155–165

Masuko T, Kashiwagi K, Kuno T, Nguyen ND, Pahk AJ, Fukuchi J, Igarashi K, Williams K (1999) A regulatory domain (R1–R2) in the amino terminus of the *N*-methyl-D-aspartate receptor: effects of spermine, protons, and ifenprodil, and structural similarity to bacterial leucine/isoleucine/valine binding protein. Mol Pharmacol 55: 957–969

Reisberg B, Doody R, Stoffler A, Schmitt F, Ferris S, Mobius HJ, for the memantine study group (2003) Memantine in moderate-to-severe Alzheimer's disease. N Engl J Med 348: 1333–1341

Rogawski MA (1992) The NMDA receptor, NMDA antagonists and epilepsy therapy. A status report. Drugs 44: 279–292

Sakurada K, Masu M, Nakanishi S (1993) Alteration of Ca^{2+} permeability and sensitivity to Mg^{2+} and channel blockers by a single amino acid substitution in the *N*-methyl-D-aspartate receptor. J Biol Chem 268: 410–415

Sobolevsky AI, Rooney L, Wollmuth LP (2002) Staggering of subunits in NMDAR channels. Biophys J 83: 3304–3314

Watanabe M, Inoue Y, Sakimura K, Mishina M (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. Neuroreport 3: 1138–1140

Williams K (1997) Interactions of polyamines with ion channels. Biochem J 325: 289–297

Wollmuth LP, Kuner T, Seeburg PH, Sakmann B (1996) Differential contribution of the NR1- and NR2A-subunits to the selectivity filter of recombinant NMDA receptor channels. J Physiol 491: 779–797

Authors' address: Prof. Keiko Kashiwagi, Faculty of Pharmacy, Chiba Institute of Science, 15-8 Shiomi-cho, Choshi, Chiba 288-0025, Japan, Fax: +81-479-30-4704, E-mail: kkashiwagi@cis.ac.jp