Form 2-4-2

Japan-US Brain Research Cooperation Program

The Group Joint Study Report [field: 1]

1. The representative of Group Joint Study:

Graduate School of Pharmaceutical Sciences, Chiba University / Professor / Kazuei Igarashi

2. The Project Title:

The structure and function of glutamate receptors and the block and modulation of these receptors by polyamines

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5. The Term of Research: From 2002. 4. 1 To 2005. 3. 31 (3 Years)

6. The Abstract, the Result and the Significance of Research (300 Words):

Structure and function of NMDA receptor has been studied using site-directed mutagenesis and electrophysiological recording. We found that the polyamines, especially spermine, stimulate the activity of NMDA receptor at the depolarization state and inhibit it at the hyperpolarization state. In addition, we also

found that a regulatory domain (NR1R, NR2AR and NR2BR) having a polyamine binding site exists in the NH_2-terminal region in addition to the agonist binding- and the channel forming-domains. The intensity of affinity of spermine binding to the regulatory domains was in the order NR1R > NR2BR > NR2AR, and that of ifenprodil was in the order NR1R > NR2BR. The binding of ifenprodil to NR2AR was not observed. To study the *in vivo* polyamine effect on NMDA receptor, the preparation of the polyamine-irresponsive NMDA receptor knock-in mice is under construction.

The properties of δ2 receptor in which agonist(s) is not yet known were found to be similar to those of NMDA receptor.

NMDA receptors are involved in neurodegeneration after ischemia and stroke, in seizure activity, and in transmission of nociceptive signals in the spinal cord. There is a great potential for the use of NMDA antagonists and modulators as neuroprotectants, anticonvulsants, and analgesics. It was found that anthraquinone spermidine (AQ34) blocked the activity of NMDA receptors specifically. The IC_{50} of AQ34 for NR1/NR2A was 7.1 μM. Using the AQ34, we found that the channel of NMDA receptor mainly consisted of the M2 loop and the M3 transmembrane segment. Experiments are in progress to develop the chemicals which modify the function of NMDA receptors specifically for clinical application using AQ34 as a lead compound.

7. The Others (Practical Issues, Special mention Matters):

Nothing in particular.

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Anthraquinone Polyamines: Novel Channel Blockers to Study *N*-Methyl-D-Aspartate Receptors

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ABSTRACT

The effects of various anthraquinone polyamines (AQP) were studied at recombinant *N*-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus laevis* oocytes. The AQP derivatives had different numbers of methylene groups between the NH_2 (or NH) groups in their spermidine-like tail. Thus, we termed these derivatives AQ33, AQ34, etc. All AQP derivatives inhibited responses of NR1/NR2 receptors in oocytes voltage-clamped at −70 mV, with IC_{50} values between 4 and 22 μM. The block was strongly voltage-dependent. AQ34 and AQ33b inhibited responses of NR1/NR2 receptors but did not inhibit responses of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expressed from GluR1 or GluR2(Q), indicating that AQ34 and AQ33b are preferential NMDA antagonists. Results of experiments using mutant NR1 and NR2 subunits identified residues that influence block by AQ34 and AQ33b. These residues are located in the outer vestibule at the selectivity filter/narrowest constriction of the channel and in the inner vestibule below the level of the selectivity filter. The results with mutant NR1 and NR2 subunits are consistent with the idea that NR1(Asn616) and NR2B(Asn616), but not NR2B(Asn615), make the narrowest constriction of NMDA channel.

N-Methyl-D-aspartate (NMDA) receptors are involved in excitatory synaptic transmission and synaptic plasticity, and they are potential targets for neuroprotective agents and anticonvulsants (Choi, 1988; Rogawski, 1992). The receptors are modulated by various endogenous and exogenous ligands. Antagonists or blockers acting at various sites, including the agonist binding sites, the ion channel pore, and extracellular allosteric sites, have been described (Dingledine et al., 1999). The receptors are probably tetramers and are composed of combinations of three types of subunits—NR1, NR2, and NR3. 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 (Hollmann and Heinemann, 1994; Dingledine et al., 1999).

The endogenous polyamine spermine has multiple effects on NMDA receptors, including stimulation that increases the size of NMDA currents 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 be NMDA channel blockers, and such compounds are useful as tools to study the structure and function of glutamate receptors (Jackson and Usherwood, 1988; Williams, 1997). The M2 loop region in NR1 and NR2 subunits is a critical determinant of divalent cation permeability and Mg^{2+} block. In particular, asparagine residues in this region form part of Mg^{2+} binding site and contribute to the selectivity filter of the channel (Dingledine et al., 1999). These asparagine residues 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-tribenzyl-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AQP, anthraquinone polyamine; AQCC, anthraquinone-2-carboxyl chloride; MK-801, dizocilpine maleate; TB34, N^1,N^4,N^8-tribenzylspermidine; AQ22, 2-{[2-[(anthraquinone-2-carbonyl)amino]ethyl]amino}ethylamine; AQ32, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}ethylamine; AQ23, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}ethylamine; AQ24, 4-{[2-[(anthraquinone-2-carbonyl)amino]ethyl]amino}butylamine; AQ33b, 4-(anthraquinone-2-carbonyl)-4-azaheptane-1,7-diamine; AQ33, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}propylamine; AQ34, 4-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}butylamine.

spermidine (TB34) (Sakurada et al., 1993; Kashiwagi et al., 2002). 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).

To study the structure of NMDA channels in more detail, we developed several polyamine derivatives such as bisethylpolyamines (Igarashi and Williams, 1995), N^1-dansylspermine (Chao et al., 1997), and benzylpolyamines (Igarashi et al., 1997). In this study, we found that anthraquinone polyamine (AQP) derivatives, which contain a larger head group than N^1-dansylspermine, also act as preferential NMDA channel blockers, and we have looked for residues in NR1 and NR2 subunits that influence block and permeation of the AQP derivatives.

Materials and Methods

NMDA Clones and Site-Directed Mutagenesis. The NR1 clone used in these studies is the NR1A variant (Moriyoshi et al., 1991), which lacks the 21-amino acid insert encoded by exon-5. This clone, and some of the NR1 mutants in the M2 and M1-M2 linker region (Sakurada et al., 1993), were gifts from Dr. S. Nakanishi (Faculty of Medicine, Kyoto University, Japan). The preparation of most other NR1 and NR2 mutants has been previously described (Kashiwagi et al., 1997, 2002; Williams et al., 1998; Masuko et al., 1999). The wild-type rat and mouse NR2B clones (Kutsuwada et al., 1992; Monyer et al., 1992) were gifts from Drs. P. H. Seeburg (Center for Molecular Biology, University of Heidelberg, Germany) and M. Mishina (University of Tokyo, Japan). Site-directed mutagenesis to construct NR2B N615G and NR2A N614G was carried out by the method of Ho et al. (1989) using the polymerase chain reaction. Amino acids are numbered from the initiator methionine in each subunit. This differs from the numbering system used in some other laboratories, in which residues are numbered from the start of the mature peptide. In the case of NR1, there is an 18-amino acid signal peptide, for example, residue NR1(Asn616) described in this study corresponds to residue NR1(Asn598) using the alternative numbering scheme (Kuner et al., 1996).

Expression in Oocytes and Voltage-Clamp Recording. The preparation of capped cRNAs and the preparation, injection, and maintenance of oocytes were carried out as described previously (Williams et al., 1993). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (0.1–4 ng of NR1 plus 0.5–20 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage clamp using a GeneClamp 500 amplifier (Axon Instruments, Union City, CA) as described previously (Williams, 1993). Electrodes were filled with 3 M KCl and had resistances of 0.4 to 4 MΩ. Oocytes were continuously superfused with a saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.5), and in most experiments, oocytes were injected with K^+-BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) (100 nl of 40 mM, pH 7.0–7.4) on the day of recording.

Data analysis and curve fitting were carried out using Axograph (Axon Instruments) or SigmaPlot (SPSS Inc., Chicago, IL) on Macintosh computers. To obtain IC_{50} values of polyamine derivatives, concentration-inhibition curves were fit to eq. 1.

$$I_{Glu+blocker}/I_{Glu} = 1/[1 + ([blocker]/IC_{50})^{nH}] \quad (1)$$

in which I_{Glu} is the response to glutamate and $I_{Glu+blocker}$ is the response to glutamate measured in the presence of the blocker.

To study the voltage dependence of block, voltage ramps were constructed by ramping the command signal from −150 to +40 mV over 6 s. Leak currents, measured in the absence of agonist and blockers, were digitally subtracted. We chose concentrations of blockers that gave a 50 to 80% inhibition at −70 mV at a particular mutant. For analysis of the voltage dependence of block by AQP derivatives, data were analyzed using the model of Woodhull (1973) by fitting the data to eq. 2.

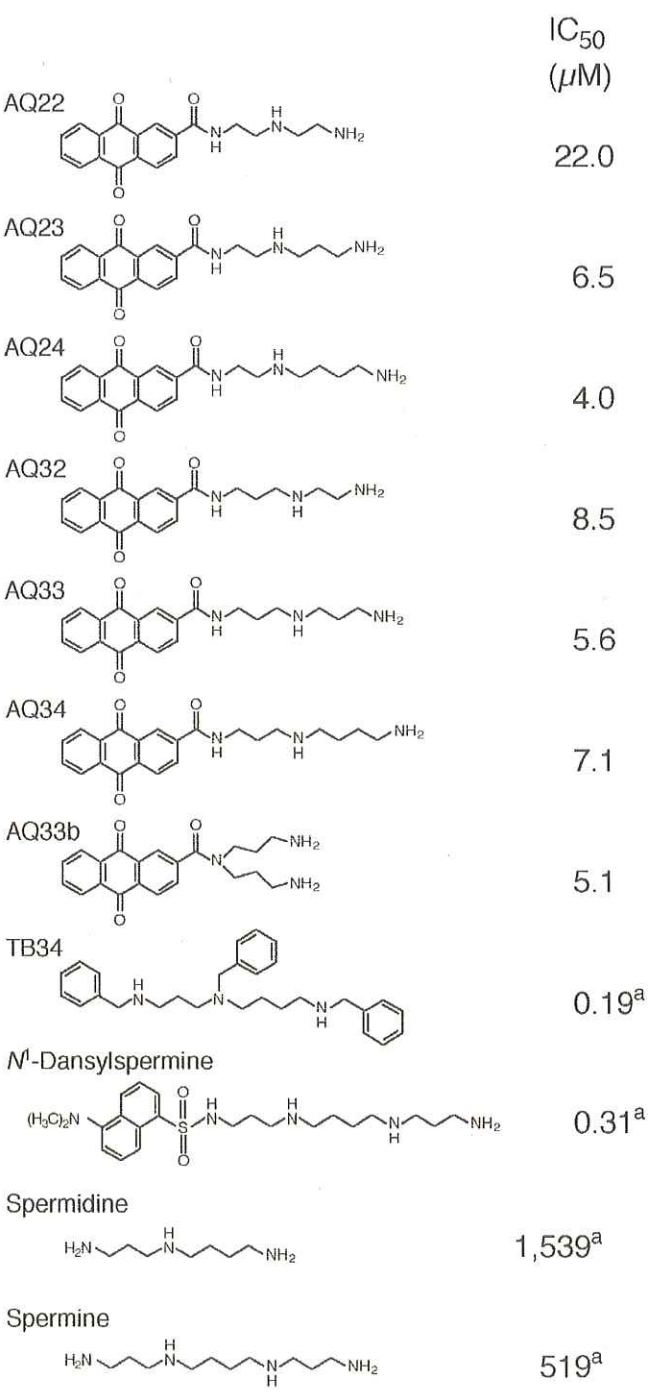

Fig. 1. Structures and activity of AQP derivatives and other polyamines. IC_{50} values were determined from concentration-inhibition curves ($n = 4$ oocytes) at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV. [a]Data for spermine, spermidine, N^1-dansylspermine, and TB34 are from Chao et al., 1997 and Igarashi et al., 1997.

$$I_{Glu+blocker}/I_{Glu} = \alpha/[1 + ([blocker]/K_d(0)\exp(z\delta FV/RT))] \quad (2)$$

in which I_{Glu} is the control response to glutamate, $I_{Glu+blocker}$ is the response to glutamate measured in the presence of the blocker, α is the fraction of voltage-dependent block, $K_d(0)$ is the half-blocking concentration at 0 mV, z is the charge of the blocker, δ is the fraction

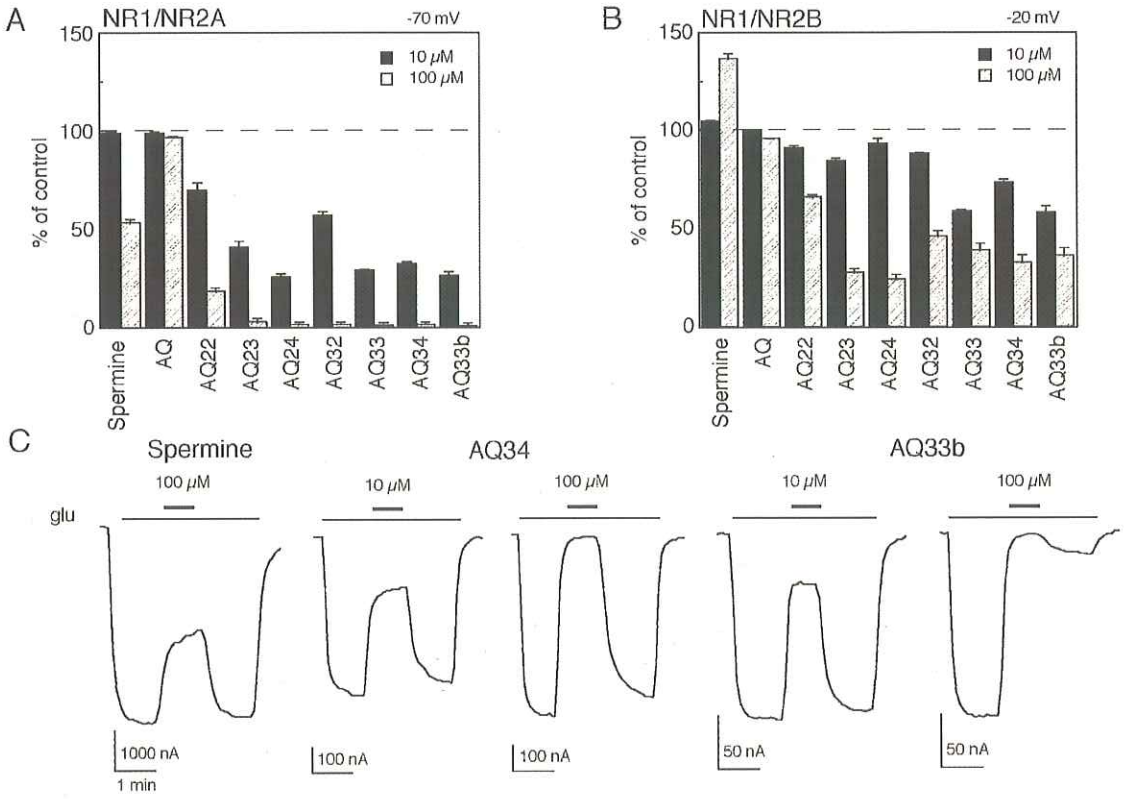

Fig. 2. Effects of AQP derivatives at NMDA receptors. The effects of AQP derivatives, each at 10 and 100 μM, on responses to glutamate (10 μM, with 10 μM glycine) were determined in oocytes expressing NR1/NR2A receptors voltage-clamped at −70 mV (A) and NR1/NR2B receptors voltage-clamped at −20 mV (B). Data are expressed as a percentage of the control response. Values are mean ± S.E.M. from four oocytes for each compound. C, representative traces showing block by spermine, AQ34, and AQ33b at NR1/NR2A receptors (Glu = 10 glutamate + 10 μM glycine). Recovery from block by 100 μM AQ33b was incomplete.

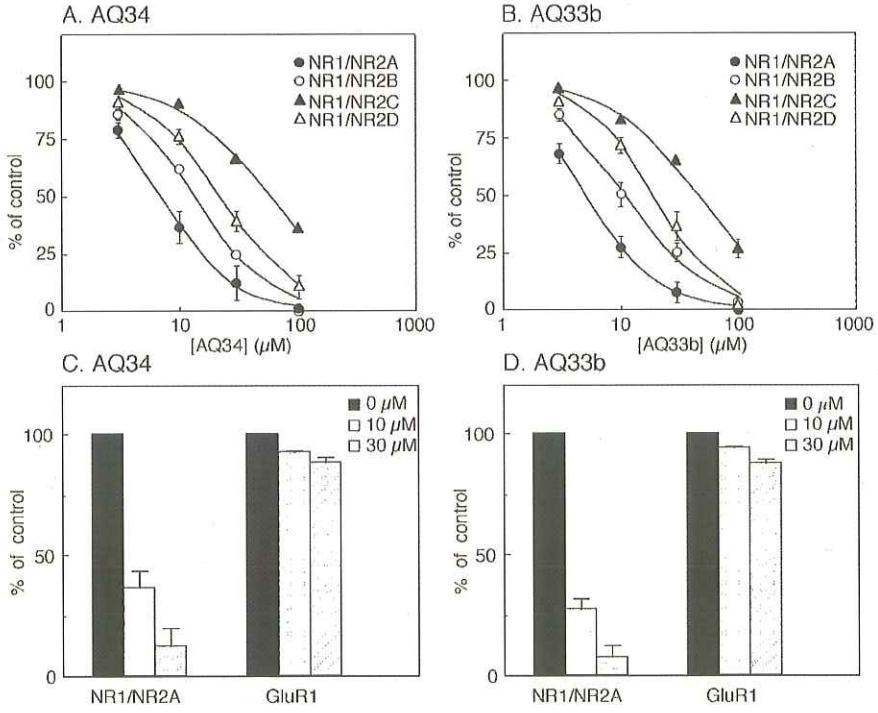

Fig. 3. Effects of AQP derivatives at NMDA and AMPA receptors. A and B, concentration-inhibition curves for AQ34 (A) and AQ33b (B) were determined at NR1/NR2 receptors containing NR2A, NR2B, NR2C, and NR2D. Values are mean ± S.E.M. from four oocytes for each subunit combination. C and D, effects of AQ34 (C) and AQ33b (D) were determined at NR1/NR2A receptors activated by glutamate (Glu, 10 μM, with 10 μM glycine) and at GluR1 receptors activated by 100 μM kainate in oocytes voltage-clamped at −70 mV. Responses to glutamate or kainate measured in the presence of AQ34 or AQ33b are expressed as a percentage of the control response at each receptor type. Values are mean ± S.E.M. from four oocytes.

of the membrane electric field sensed by the blocker at its binding site within that field, and F, R, and T have their usual meanings. In the fitting procedure, the parameters α, $K_d(0)$, and $z\delta$ were free. The α function was included in eq. 2 because, in some cells, the glutamate response showed a small run-down or run-up over time. The inclusion of the α variable improves the fitting procedure.

Synthesis of Anthraquinone Polyamines. The polyamine derivatives used in this study are shown in Fig. 1. AQ22 and AQ32 were synthesized from anthraquinone-2-carboxyl chloride (AQCC) according to the method of Okawara et al. (1990). AQ23 and AQ24 were prepared by treatment of protected triamines (*N*-[2-benzyloxy carbonylamino]ethyl)-*N,N'*-bis(*tert*-butoxycarbonyl)-diamine) with AQCC, and the Boc (*tert*-butoxycarbonyl) group was removed. AQ33b was prepared through phthalation of triamine followed by reaction with AQCC, and the phthaloyl group was removed. AQ33 and AQ34 were prepared from 1-(anthraquinone-2-carboxyl)amino-3-bromopropane and diamine. The structures of each compound were determined by spectral data and elemental analysis.

Results

Activities of AQP Derivatives. Polyamines, such as spermine, have various effects on NMDA receptors including stimulation and voltage-dependent block, which are dependent on the subunit composition of the receptors (Williams et al., 1994; Zhang et al., 1994). The various effects can be studied in relative isolation by the use of particular subunit combinations, agonist concentrations, and holding potentials. NR1/NR2B, but not NR1/NR2A, receptors are potentiated by spermine in the presence of saturating concentrations of agonist, whereas both subtypes are blocked in a voltage-dependent manner by spermine (Williams, 1994; Williams et al., 1994). Thus, to look for voltage-dependent block, AQP derivatives were studied at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV. To look for stimulation, we measured effects at NR1/NR2B receptors voltage-clamped at −20 mV. At a concentration of 10 μM, all AQP derivatives inhibited responses to glutamate at NR1/NR2A receptors, and strong inhibition was observed with AQ23, AQ24, AQ33, AQ34, and AQ33b (Fig. 2A). By itself, the AQP head group AQ-2 sulfonic acid (AQ), was inactive indicating that the polyamine tail is essential for inhibitory activity. At NR1/NR2B receptors, the AQP derivatives showed inhibition but not potentiation of responses to glutamate (Fig. 2B). It is possible that potentiation by these analogs is masked by a potent, voltage-dependent channel block that is manifest even at −20 mV, but results of studies using voltage ramps (see below) suggests that this is not the case because stimulation was not seen even at depolarized potentials. Thus, unlike spermidine and spermine, AQP derivatives do not potentiate NR1/NR2B receptors. The AQP derivatives had IC_{50} values of 4 to 22 μM at NR1/NR2A receptors (Fig. 1).

The effects of two AQP derivatives, AQ34 and AQ33b, were characterized in detail. To determine the subunit selectivity of block by AQ34 and AQ33b at NMDA receptors, we measured concentration-inhibition curves at NR1/NR2 receptors containing the NR2A, NR2B, NR2C, and NR2D subunits (Fig. 3, A and B). For both AQ34 and AQ33b, there were significant differences in sensitivity between receptors containing various NR2 subunits. For both compounds, the largest difference (8- to 9-fold) was between NR1/NR2A and NR1/NR2C. To determine whether AQ34 and AQ33b were selective NMDA receptor antagonists, we studied their effects at AMPA receptors expressed from the GluR1 subunit and activated by the nondesensitizing agonist kainate (Fig. 3, C and D). At concentrations of 10 to 30 μM, which produce substantial inhibition at NMDA receptors, AQ34 and AQ33b had little effect at GluR1 receptors. Similar effects were seen at homomeric GluR2(Q) receptors, at which 10 μM AQ34 inhibited responses by 13 ± 1% (n = 9). Thus, the AQP derivatives preferentially block NMDA receptors.

Identification of Amino Acid Residues Involved in Block by AQP Derivatives. We carried out experiments to identify amino acid residues that are involved in the block of NMDA channels by AQ34 and AQ33b using a series of NR1 and NR2B mutants that we have previously generated to study the pore and vestibule structure in NMDA receptors

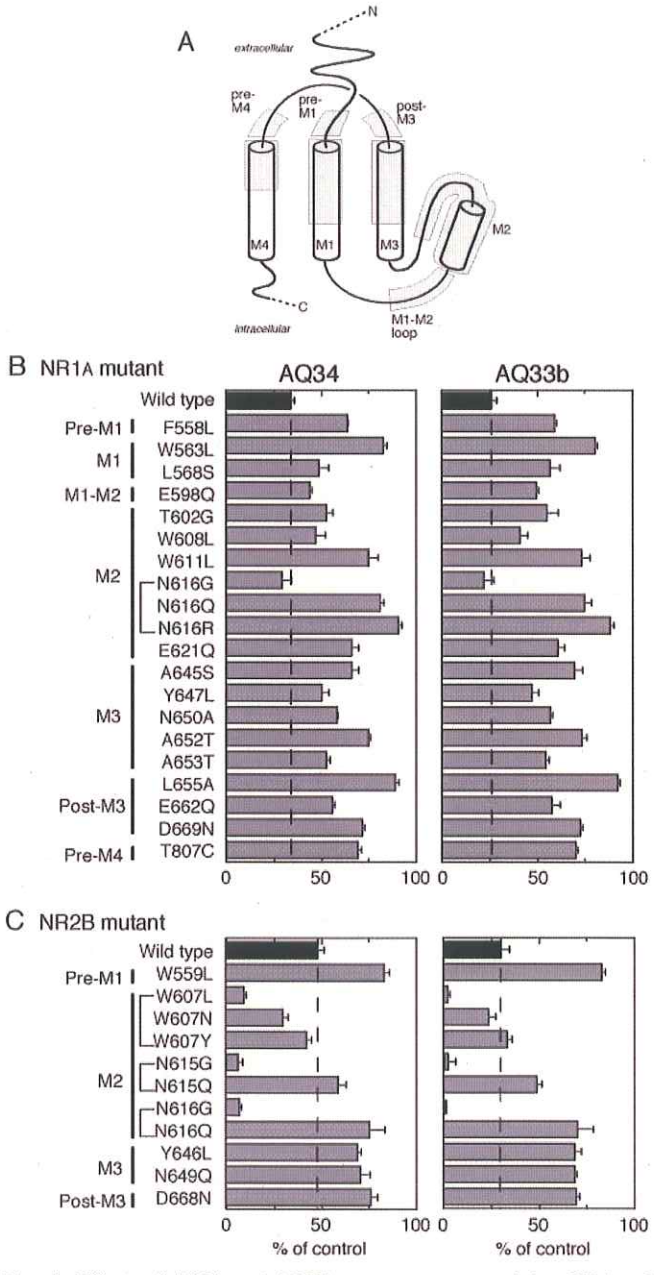

Fig. 4. Effects of AQ34 and AQ33b at receptors containing NR1 and NR2B mutants. A, schematic of the NR1 subunit, which contains three transmembrane segments (M1, M3, and M4), a re-entrant loop (M2), and a large extracellular loop between M3 and M4. The stippled boxes indicate the region in which mutations were studied. B, the effects of AQ34 and AQ33b (10 μM) were determined in oocytes expressing wild-type and mutant NR1/NR2A receptors and voltage-clamped at −70 mV. C, the effects of AQ34 and AQ33b at 10 μM were determined in oocytes expressing wild-type and mutant NR1/NR2B receptors and voltage-clamped at −70 mV. Values are mean ± S.E.M. from four oocytes for each mutant.

(Kashiwagi et al., 1997, 2002; Williams et al., 1998). We recently identified residues that differentially affect block by memantine, MK-801, and the polyamine derivative TB34 (Kashiwagi et al., 2002). TB34 (Fig. 1) is about 25-fold more potent than AQ34 and AQ33b at NR1/NR2A receptors (Igarashi et al., 1997), more hydrophobic than the parent compound spermidine, and still a highly flexible molecule but lacks a free polyamine "tail" as found in the AQP derivatives. Thus, the effects of 10 μM AQ34 and AQ33b were measured using NR1 and NR2B mutants which affect block by spermine, TB34, and NR1 mutants in the inner vestibule below the level of selectivity filter (Fig. 4). Of the amino acid residues tested, most mutations which reduced block by TB34 (Kashiwagi et al., 1997, 2002) also reduced block by AQ34 and AQ33b; however, mutations at a number of additional residues (NR1, Leu568, Glu598, Thr602, Trp608, and Trp611) reduced block by the AQP derivatives but not by TB34 (Kashiwagi et al., 2002). These amino acid residues are mainly located in the inner vestibule below the level of selectivity filter. The differential effects of these mutants on block by TB34 and AQP may be due to differences in the size and hydrophobicity of the anthraquinone and benzene substitutions of these two classes of polyamines and/or to the presence of a "free" polyamine tail with unsubstituted amino groups in the AQP derivatives.

Residues at the Selectivity Filter. Mutations at the critical asparagine in the M2 loop of NR1 (N616Q and N616R) reduced block by AQ34 and AQ33b (Fig. 4); however, substitution of Gly for Asn slightly enhanced the block by AQ34 and AQ33b. Similar results were obtained with N^1-dansylspermine, which has an intact polyamine tail with a hydrophobic head group (Chao et al., 1997). 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 sites (Dingledine et al., 1999). Substitution of Gln for Asn at either position reduced block by AQ34 and AQ33b, whereas substitution of Gly for Asn increased block (Fig. 4). A tryptophan residue in the M2 loop of NR2B (Trp607) has also been proposed to contribute directly to the selectivity filter or to markedly influence the structure and position of the selectivity filter and the binding site for extracellular Mg^{2+} (Williams et al., 1998). Mutations at NR2B(Trp607) to Leu or Asn also increased block by AQ34 and AQ33b (Fig. 4C).

When mutations were made at NR1(Asn616), NR2B-(Trp607), NR2B(Asn615), and NR2B(Asn616) to reduce the size of the side chain (Asn to Gly or Trp to Leu), in all cases sensitivity to AQ34 increased (Fig. 5). The sensitivity of a number of double mutants was examined by coexpressing NR1(N616G) with each of several NR2B mutants (Fig. 5D). The sensitivity to AQ34 was nearly equal in the three double mutants, and it was slightly higher than that of the single mutants NR2B(W607L) and NR2B(N616G). The sensitivity of the double NR1(N616G)/NR2B(N615G) mutant was less than that of the single NR2B(N615G) mutant, possibly due to increased permeation of AQ34 through the channel in the double mutant (see below). Similar results were seen with mutations at corresponding positions in NR2A, although the effect of the NR2A(W606L) mutant was less than that of the corresponding NR2B(W607L) mutant (Fig. 5, C and E).

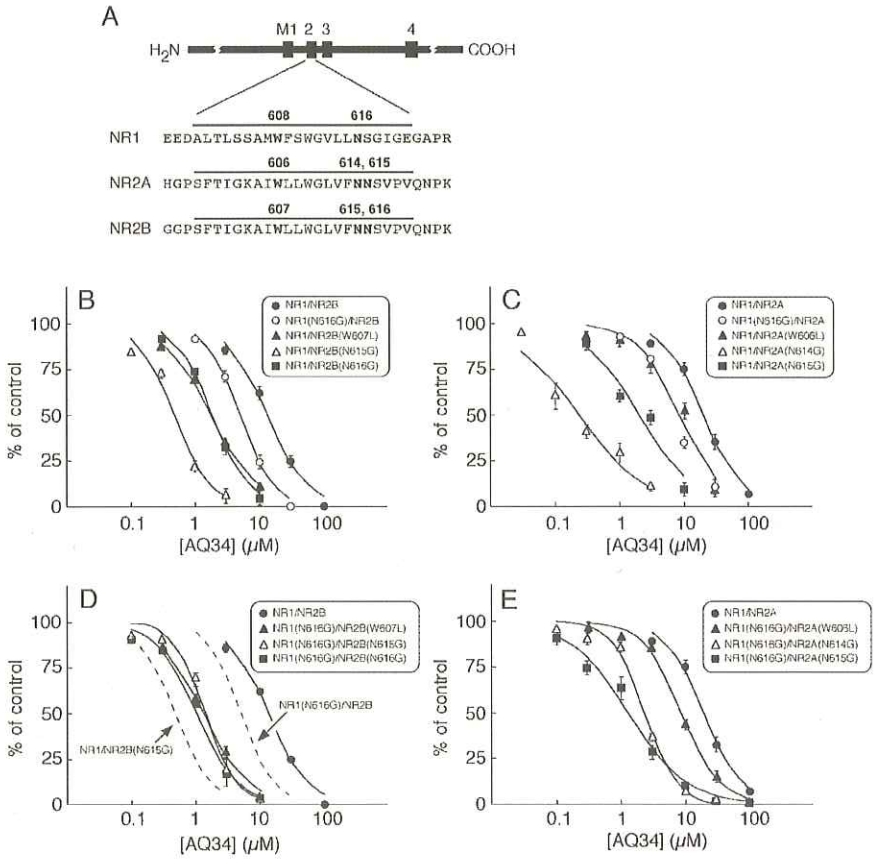

Fig. 5. Effects of AQ34 on NMDA receptors containing mutations at the selectivity filter. The positions of the M1-M4 segments of NMDA receptor and amino acid sequences of the M2 region of NR1, NR2A, and NR2B are shown in panel A. Concentration-inhibition curves for AQ34 were determined at NR1/NR2B (B and D) receptors and NR1/NR2A receptors (C and E) containing single or double mutations and voltage-clamped at −70 mV. Values are mean ± S.E.M. from four oocytes for each mutant.

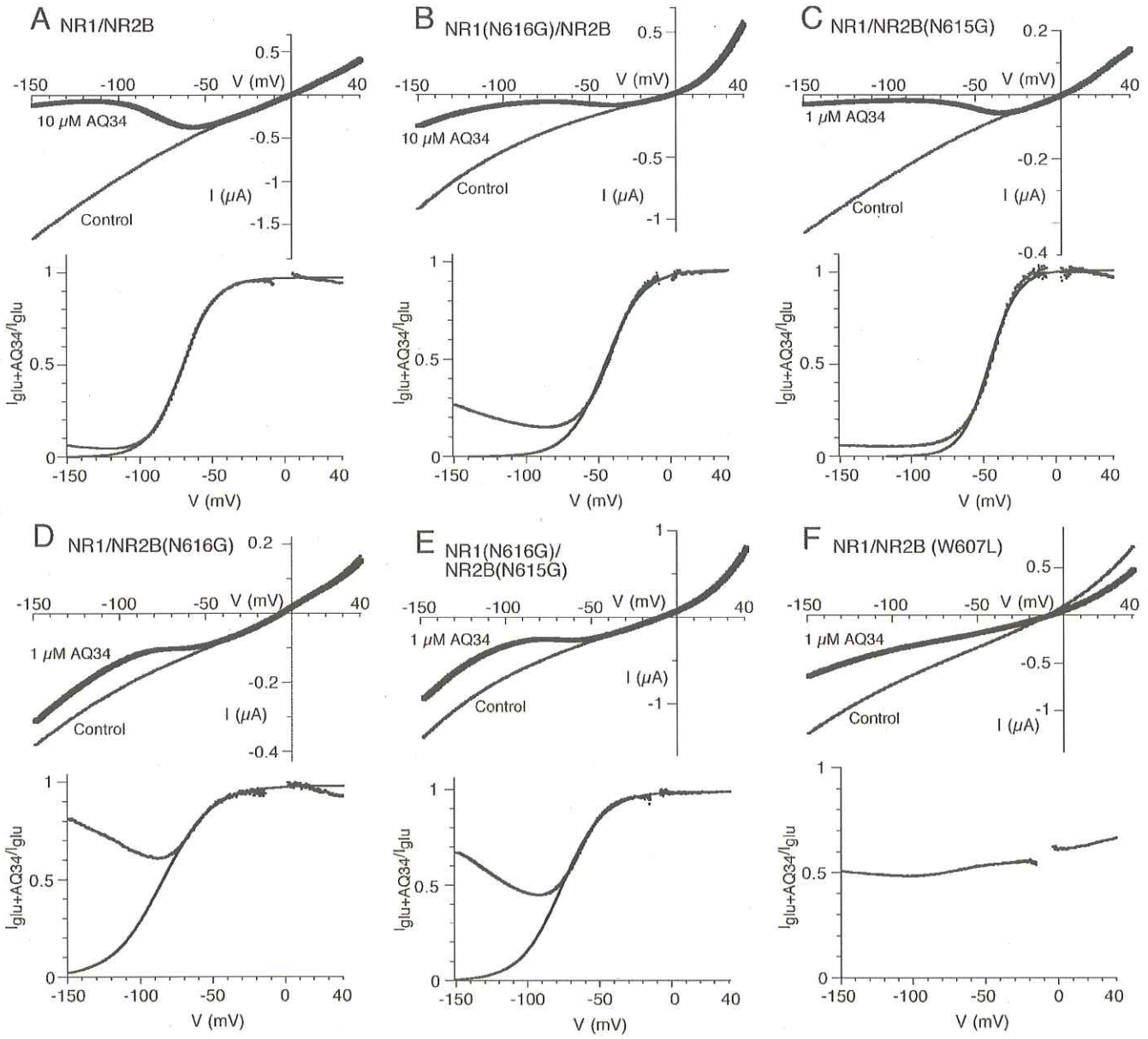

Fig. 6. Block and permeation of AQ34 at mutant NMDA channels. Top panels, I-V curves were constructed by voltage ramps from −150 to +40 mV in oocytes expressing wild-type and mutant receptors. Responses to 10 μM glutamate (with 10 μM glycine) were measured in the absence (control) and presence of AQ34. Leak currents were subtracted. Bottom panels, currents measured in the presence of AQ34 ($I_{Glu+AQ34}$) are expressed as a fraction of the control current (I_{Glu}) for each oocyte. Data around the reversal potential have been masked. The solid lines are fits to the Woodhull model (eq. 2). Note the incomplete block at some mutant NMDA receptors and the apparent reversal of block and restoration of inward current at very negative potentials. At NR1/NR2B(W607L) (F), block by 1 μM AQ34 showed no apparent voltage dependence and was not relieved even at positive membrane potentials. Similar effects were seen with 0.3 to 10 μM AQ34 at NR1/NR2B(W607L) receptors (data not shown).

Block of NMDA channels by spermine and by a number of polyamine analogs is voltage-dependent, but the degree of voltage dependence and/or the depth of the binding site varies between different polyamine analogs. For example, N^1-dansylspermine ($z\delta$ = 2.58; assuming z = 4 then δ = 0.65) shows a stronger voltage dependence than TB34 ($z\delta$ = 1.38; assuming z = 3 then δ = 0.46) (Chao et al., 1997; Kashiwagi et al., 2002). Some mutations at the selectivity filter have been shown to alter the apparent permeation of polyamines and polyamine derivatives. Thus, we carried out experiments to study the voltage dependence and permeation of AQ34 and the effects of mutations at the selectivity filter on block and permeation. The purpose of these experiments was 2-fold: first, to study voltage dependence using the Woodhull (1973) model of voltage-dependent block to determine values for the $K_d(0)$ and for the apparent valence, $z\delta$; and second, to determine whether AQ34 shows significant permeation of the channel manifest as a partial relief of block and restoration of an inward current at very negative membrane potentials. As an index of the degree of permeation, we calculated the ratio of the fractional current in the presence of AQ34 at −130 mV compared with the fractional current in the presence of AQ at −70 mV ($F_{-130/-70}$). These two voltages were chosen arbitrarily but are within the range where AQ34 produces a

TABLE 1

Effects of NR1 and NR2 mutants on block by AQ34

Values for $K_d(0)$ and $z\delta$ were determined using voltage ramps analyzed with the Woodhull model of voltage-dependent block. $F_{-130/-70}$ is the ratio of the fractional current in the presence of AQ34 at −130 and −70 mV. Data are the mean ± S.E.M.

Receptor	$K_d(0)$	$z\delta$	$F_{-130/-70}$	n
	μM			
Wild-type NR/NR2B	3481 ± 205	2.12 ± 0.02	0.10 ± 0.01	29
NR1(N616G)/NR2B	344 ± 29	2.00 ± 0.05	1.23 ± 0.05	4
NR1/NR2B(N615G)	216 ± 40	2.77 ± 0.05	0.30 ± 0.15	12
NR1/NR2B(N616G)	190 ± 39	1.75 ± 0.09	1.31 ± 0.10	10
NR1(N616G)/NR2B(N615G)	345 ± 172	2.12 ± 0.16	0.80 ± 0.11	4
NR1(N616G)/NR2B(N616G)	9 ± 24	2.09 ± 0.11	1.62 ± 0.04	7
NR1/NR2B(W607L)			1.00 ± 0.01	11
NR1/NR2B(W610L)	4381 ± 331	2.21 ± 0.05	0.14 ± 0.01	5
Wild-type NR1/NR2A	5450 ± 1482	2.31 ± 0.05	0.08 ± 0.02	13
NR1/NR2A(N614G)	180 ± 42	2.62 ± 0.10	0.12 ± 0.04	7
NR1/NR2A(N615G)	142 ± 44	1.83 ± 0.07	1.11 ± 0.08	12
NR1(N616G)/NR2A(N614G)	353 ± 89	1.97 ± 0.11	0.92 ± 0.04	5
NR1(N616G)/NR2A(N615G)	169 ± 22	2.24 ± 0.08	1.59 ± 0.05	8
NR1/NR2A(W606L)	672 ± 237	1.68 ± 0.09	0.86 ± 0.02	5
NR1/NR2A(W609L)	4362 ± 797	2.21 ± 0.05	0.13 ± 0.04	6

n, number of oocytes used.

strong block at wild-type receptors. At wild-type receptors, for a strongly voltage-dependent blocker that does not permeate the channel, there will be a much greater block at −130 compared with −70 mV. Thus, the value of $F_{-130/-70}$ will be much smaller than 1.0. If the blocker shows significant permeation with a particular mutant, there will be less of an increase in the degree of block between −130 and −70 mV, and the value of $F_{-130/-70}$ will be greater than the wild-type and will approach 1.0. When there is dramatic permeation, there may actually be less block at −130 than at −70 mV, and the value of $F_{-130/-70}$ will be greater than 1.0. An increase in permeation may reflect an increase in the size of the narrow constriction at the selectivity filter.

At wild-type NR1/NR2B and NR1/NR2A receptors, block by AQ34 was strongly voltage-dependent with a $z\delta$ value of 2.1 to 2.3 and a $K_d(0)$ value of 3.4 to 5.4 mM (Fig. 6A; Table 1). Block of wild-type receptors was almost complete with little permeation ($F_{-130/-70}$ = 0.08–0.10) at very negative membrane potentials. There was a small increase in permeation with Asn to Gly mutation at NR1(N616G) and a large increase in permeation with the NR2B(N616G) mutant (Fig. 6, B and D; Table 1). Enhanced permeation was also seen with the double mutations NR1(N616G)/NR2B(N615G) and NR1(N616G)/NR2B(N616G) (Table 1). The results suggest that NR1(Asn616) and NR2B(Asn616) are the key determinants of the narrowest part of the channel. Similar results were obtained with NR2A mutants. Thus, AQ34 could easily permeate NR1/NR2A(N615G), NR1(N616G)/NR2A(N614G), and NR1(N616G)/NR2A(N615G) but not NR1/NR2A(N614G) (Table 1). The NR1 and NR2 mutations primarily affected the $K_d(0)$ suggesting an increased affinity of binding although, in some cases, there were changes in $z\delta$ (Table 1). This could reflect a shift in the depth of the binding site due to the mutation, but the incomplete block may also distort the Woodhull (1973) fit, as we could only fit the initial component of block in mutants where there was considerable breakthrough at very negative potentials (see lower panels in Fig. 6, D and E).

The NR2B(W607L) mutant increased the potency of AQ34 (Fig. 5B); however, block of NR1/NR2B(W607L) receptors by AQ34 showed no apparent voltage dependence (Fig. 6F). This is a surprising result, and it may suggest that NR2B(Trp607) is important for maintenance of the structure of the channel.

Discussion

Glutamate receptor-mediated neurotoxicity contributes to a variety of neurological disorders as well as cell death following trauma and stroke. Development of clinically useful compounds is therefore important, especially given that few glutamate receptor blockers simultaneously satisfy the requirements of effectiveness and safety or lack of side effects. In this regard, 2,3-benzodiazepines and amino-alkyl-cyclohexanes have been recently developed as glutamate receptor antagonists (Rogawski, 1993; Parsons et al., 1999). Polyamine derivatives are also potential lead compounds for novel, therapeutically useful NMDA antagonists and are useful as tools to study NMDA and other glutamate receptors. In this study, we have found that AQP derivatives preferentially block NMDA receptors. The AQP derivatives are all triamines and were 70- to 400-fold more potent than the natural triamine spermidine but about 10-fold less potent than N^1-dansylspermine and TB34. N^1-Dansylspermine blocks NMDA channels and is also a potent and poorly reversible antagonist at AMPA [GluR1 or GluR2(Q)] and δ2 glutamate receptors (Williams et al., 2003) whereas TB34, like the AQP derivatives, is a selective NMDA receptor blocker (Igarashi et al., 1997). This indicates that the nature of the head group on these relatively simple polyamine derivatives is an important determinant of channel selectivity, presumably reflecting differences in the interaction with different residues in NMDA and AMPA channels or differences in the accessibility of the binding sites within these channels.

We have recently constructed a model of the NMDA channel pore and vestibule regions (Kashiwagi et al., 2002) based on the results of mutagenesis studies and results from previous studies in which solvent-accessible residues were probed after cysteine substitution (Kuner et al., 1996; Beck et al., 1999) and crystallographic studies describing the structure of a bacterial K^+ channel (Doyle et al., 1998). We have used this model to map the amino acid residues at which mutations influence block by AQP and to compare those

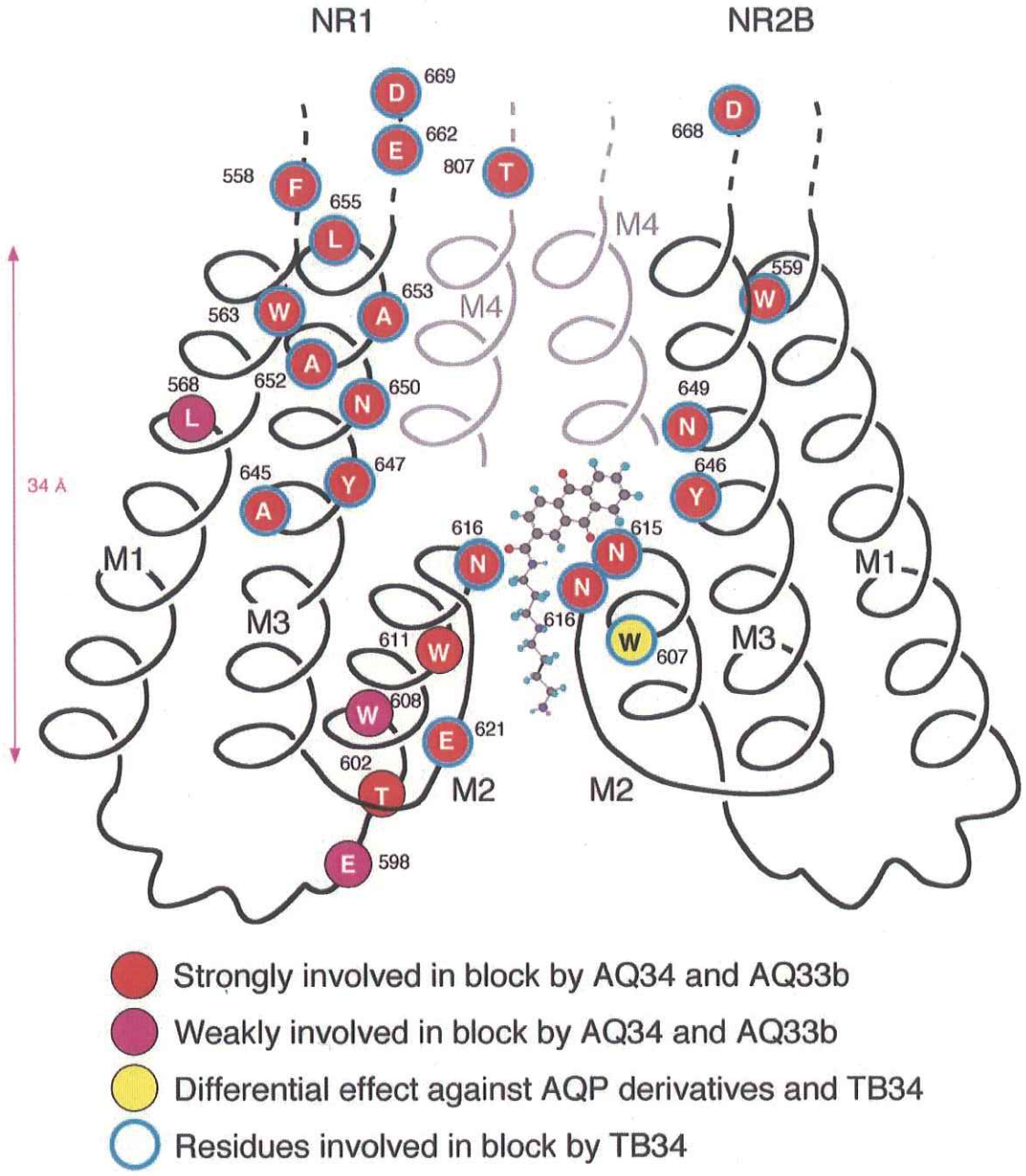

Fig. 7. Modeling residues that affect block by AQ34 and AQ33b in the pore and vestibule of the NMDA receptor. The M1-M2-M3 region is depicted as a helix-pore-loop helix similar to the structure reported for the KcsA potassium channel from *Streptomyces lividans* (Doyle et al., 1998). The M2 region contains a helix followed by a random coil structure. Red circles indicate residues at which mutations strongly affect block by AQ34 and AQ33b. Pink circles indicate residues at which mutations weakly affect block by AQ34 and AQ33b. The yellow circle indicates a residue at which mutations have differential effects on block by AQP derivatives and TB34. Circles outlined in light blue indicate residues at which mutations strongly affect block by TB34 (Kashiwagi et al., 2002).

residues with mutations that affect block by TB34 (Fig. 7). Residues that influence AQ34 and AQ33b are located mainly on the M2 loop and the M3 transmembrane segment, similar to residues that affect block by TB34; however, some residues that affect block by AQP derivatives did not influence TB34. These residues include NR1, Leu568, Glu598, Thr602, Trp608, and Trp611 (Fig. 7). Notably, four of these residues are located below the level of the selectivity filter. It is conceivable that the polyamine tail in AQ34 normally passes through the filter and interacts with these residues, even though the head group cannot easily permeate the narrow constriction (Fig. 7). In contrast, TB34 is too bulky to even partially pass through the narrow constriction in wild-type channels (Kashiwagi et al., 2002). This would also be consistent with the idea that the binding site for AQ34 ($z\delta = 2.12$; assuming $z = 3$ then $\delta = 0.71$) is deeper in the channel pore than is the site for TB34 ($z\delta = 1.38$; assuming $z = 3$ then $\delta = 0.46$). Consistent with this idea, there is a report that the adamantane derivatives IEM-1754 and IEM-1857, which are NMDA receptor channel blockers and have an amine tail,

have a binding site below the level of the selectivity filter (Antonov et al., 1998). It has been also reported that a number of residues in the intracellular vestibule of non-NMDA receptors are involved in the binding of polyamines (Panchenko et al., 1999; 2001). It also remains possible that these mutations in these regions nonspecifically disrupt the structure of the channel. Indeed, it may be that the structure of the channel is changed by substitution of Leu for Trp at NR2B(Trp607) as discussed below.

If the binding site for AQ34 lies deep within the channel near the narrow constriction (Fig. 7), then why do mutations at more peripheral residues in M1 and M3 (e.g., Asp669, Leu655, Trp563, etc. in NR1) affect block by AQ34? One possibility is that these residues affect the accessibility of AQ34 for its binding site rather than contributing to the binding site per se. That is, these residues may be involved in gating of the channel or may control access of the blocker as it passes into the channel. Consistent with this idea, many of the peripheral residues in the channel mouth that affect block by AQP derivatives also affect block by MK-801, a relatively small and rigid molecule that probably binds deep within the pore near the selectivity filter (Kashiwagi et al., 2002). Portions of the external vestibule, in particular the M3 segment, appear to undergo significant movement during gating, and the relative positions of these segments are staggered between NR1 and NR2 subunits (Sobolevsky et al., 2002a,b). A staggering of these regions of the external vestibule may account for differences in the effects of mutations at homologous positions in NR1 and NR2 subunits, particularly if these regions affect the accessibility of channel blockers to their binding site near the selectivity filter. Our data may also be consistent with the proposal that a second external binding site for NMDA receptor channel blockers exists in addition to a deep site near the selectivity filter (Subramaniam et al., 1996; Sobolevsky and Koshelev, 1998; Bolshakov et al., 2003).

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 NR2B(Asn615) also contributes to the selectivity filter (Kuner et al., 1996; Wollmuth et al., 1996; Dingledine et al., 1999). A similar relationship was observed for the corresponding residues (Asn614 and Asn615) in NR2A. The results from studies using voltage ramps suggest that NR2B(Trp607) also plays an important role to maintain the structure of channel pore and narrow constriction. This residue has previously been shown to have a profound influence on block and permeation of TB34, MK-801, Mg^{2+}, and several other blockers (Williams et al., 1998; Kashiwagi et al., 2002). Notably, the effects of mutations at NR2B(Trp607) were markedly different for AQ34 compared with TB34. For example, with NR2B(W607L) the equilibrium block by AQ34 is increased (the IC_{50} is decreased), whereas the equilibrium block by TB34 is greatly reduced (Kashiwagi et al., 2002). Block by AQ34 shows little voltage dependence in receptors containing the NR2B(W607L) mutant. It may be that the blocker accumulates near the intracellular mouth of the channel but does not easily pass back through the channel. Thus, mutations at NR2B(Trp607) may eliminate the deep binding site for AQ34 leaving only a voltage-independent binding site near the intracellular or extracellular mouth of the channel. There is a tryptophan, Trp606, at the analogous position in NR2A, but an NR2A(W606L) mutation had more modest effects than the corresponding NR2B(W607L) mutation. There may be differences in the positions of these Trp residues in NR2A and NR2B relative to the critical Asn residues in the M2 loop, which could explain the more drastic effects of mutations in NR2B as compared with NR2A. In this context, the NR2B(W607L) mutation was previously shown to have a much greater effect on block and permeation of Mg^{2+} than the corresponding NR2A(W606L) mutation (Williams et al., 1998).

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Anthraquinone Polyamines: Novel Channel Blockers to Study *N*-Methyl-D-Aspartate Receptors

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ABSTRACT

The effects of various anthraquinone polyamines (AQP) were studied at recombinant *N*-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus laevis* oocytes. The AQP derivatives had different numbers of methylene groups between the NH_2 (or NH) groups in their spermidine-like tail. Thus, we termed these derivatives AQ33, AQ34, etc. All AQP derivatives inhibited responses of NR1/NR2 receptors in oocytes voltage-clamped at −70 mV, with IC_{50} values between 4 and 22 μM. The block was strongly voltage-dependent. AQ34 and AQ33b inhibited responses of NR1/NR2 receptors but did not inhibit responses of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expressed from GluR1 or GluR2(Q), indicating that AQ34 and AQ33b are preferential NMDA antagonists. Results of experiments using mutant NR1 and NR2 subunits identified residues that influence block by AQ34 and AQ33b. These residues are located in the outer vestibule at the selectivity filter/narrowest constriction of the channel and in the inner vestibule below the level of the selectivity filter. The results with mutant NR1 and NR2 subunits are consistent with the idea that NR1(Asn616) and NR2B(Asn616), but not NR2B(Asn615), make the narrowest constriction of NMDA channel.

N-Methyl-D-aspartate (NMDA) receptors are involved in excitatory synaptic transmission and synaptic plasticity, and they are potential targets for neuroprotective agents and anticonvulsants (Choi, 1988; Rogawski, 1992). The receptors are modulated by various endogenous and exogenous ligands. Antagonists or blockers acting at various sites, including the agonist binding sites, the ion channel pore, and extracellular allosteric sites, have been described (Dingledine et al., 1999). The receptors are probably tetramers and are composed of combinations of three types of subunits—NR1, NR2, and NR3. 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 (Hollmann and Heinemann, 1994; Dingledine et al., 1999).

The endogenous polyamine spermine has multiple effects on NMDA receptors, including stimulation that increases the size of NMDA currents 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 be NMDA channel blockers, and such compounds are useful as tools to study the structure and function of glutamate receptors (Jackson and Usherwood, 1988; Williams, 1997). The M2 loop region in NR1 and NR2 subunits is a critical determinant of divalent cation permeability and Mg^{2+} block. In particular, asparagine residues in this region form part of Mg^{2+} binding site and contribute to the selectivity filter of the channel (Dingledine et al., 1999). These asparagine residues 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-tribenzyl-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AQP, anthraquinone polyamine; AQCC, anthraquinone-2-carboxyl chloride; MK-801, dizocilpine maleate; TB34, N^1,N^4,N^8-tribenzylspermidine; AQ22, 2-{[2-[(anthraquinone-2-carbonyl)amino]ethyl]amino}ethylamine; AQ32, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}ethylamine; AQ23, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}ethylamine; AQ24, 4-{[2-[(anthraquinone-2-carbonyl)amino]ethyl]amino}butylamine; AQ33b, 4-(anthraquinone-2-carbonyl)-4-azaheptane-1,7-diamine; AQ33, 3-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}propylamine; AQ34, 4-{[3-[(anthraquinone-2-carbonyl)amino]propyl]amino}butylamine.

spermidine (TB34) (Sakurada et al., 1993; Kashiwagi et al., 2002). 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).

To study the structure of NMDA channels in more detail, we developed several polyamine derivatives such as bisethylpolyamines (Igarashi and Williams, 1995), N^1-dansylspermine (Chao et al., 1997), and benzylpolyamines (Igarashi et al., 1997). In this study, we found that anthraquinone polyamine (AQP) derivatives, which contain a larger head group than N^1-dansylspermine, also act as preferential NMDA channel blockers, and we have looked for residues in NR1 and NR2 subunits that influence block and permeation of the AQP derivatives.

Materials and Methods

NMDA Clones and Site-Directed Mutagenesis. The NR1 clone used in these studies is the NR1A variant (Moriyoshi et al., 1991), which lacks the 21-amino acid insert encoded by exon-5. This clone, and some of the NR1 mutants in the M2 and M1-M2 linker region (Sakurada et al., 1993), were gifts from Dr. S. Nakanishi (Faculty of Medicine, Kyoto University, Japan). The preparation of most other NR1 and NR2 mutants has been previously described (Kashiwagi et al., 1997, 2002; Williams et al., 1998; Masuko et al., 1999). The wild-type rat and mouse NR2B clones (Kutsuwada et al., 1992; Monyer et al., 1992) were gifts from Drs. P. H. Seeburg (Center for Molecular Biology, University of Heidelberg, Germany) and M. Mishina (University of Tokyo, Japan). Site-directed mutagenesis to construct NR2B N615G and NR2A N614G was carried out by the method of Ho et al. (1989) using the polymerase chain reaction. Amino acids are numbered from the initiator methionine in each subunit. This differs from the numbering system used in some other laboratories, in which residues are numbered from the start of the mature peptide. In the case of NR1, there is an 18-amino acid signal peptide, for example, residue NR1(Asn616) described in this study corresponds to residue NR1(Asn598) using the alternative numbering scheme (Kuner et al., 1996).

Expression in Oocytes and Voltage-Clamp Recording. The preparation of capped cRNAs and the preparation, injection, and maintenance of oocytes were carried out as described previously (Williams et al., 1993). Oocytes were injected with NR1 plus NR2 cRNAs in a ratio of 1:5 (0.1–4 ng of NR1 plus 0.5–20 ng of NR2). Macroscopic currents were recorded with a two-electrode voltage clamp using a GeneClamp 500 amplifier (Axon Instruments, Union City, CA) as described previously (Williams, 1993). Electrodes were filled with 3 M KCl and had resistances of 0.4 to 4 MΩ. Oocytes were continuously superfused with a saline solution (100 mM NaCl, 2 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.5), and in most experiments, oocytes were injected with K^+-BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) (100 nl of 40 mM, pH 7.0–7.4) on the day of recording.

Data analysis and curve fitting were carried out using Axograph (Axon Instruments) or SigmaPlot (SPSS Inc., Chicago, IL) on Macintosh computers. To obtain IC_{50} values of polyamine derivatives, concentration-inhibition curves were fit to eq. 1.

$$I_{Glu+blocker}/I_{Glu} = 1/[1 + ([blocker]/IC_{50})^{nH}] \quad (1)$$

in which I_{Glu} is the response to glutamate and $I_{Glu+blocker}$ is the response to glutamate measured in the presence of the blocker.

To study the voltage dependence of block, voltage ramps were constructed by ramping the command signal from −150 to +40 mV over 6 s. Leak currents, measured in the absence of agonist and blockers, were digitally subtracted. We chose concentrations of blockers that gave a 50 to 80% inhibition at −70 mV at a particular mutant. For analysis of the voltage dependence of block by AQP derivatives, data were analyzed using the model of Woodhull (1973) by fitting the data to eq. 2.

$$I_{Glu+blocker}/I_{Glu} = \alpha/[1 + ([blocker]/K_d(0)\exp(z\delta FV/RT))] \quad (2)$$

in which I_{Glu} is the control response to glutamate, $I_{Glu+blocker}$ is the response to glutamate measured in the presence of the blocker, α is the fraction of voltage-dependent block, $K_d(0)$ is the half-blocking concentration at 0 mV, z is the charge of the blocker, δ is the fraction

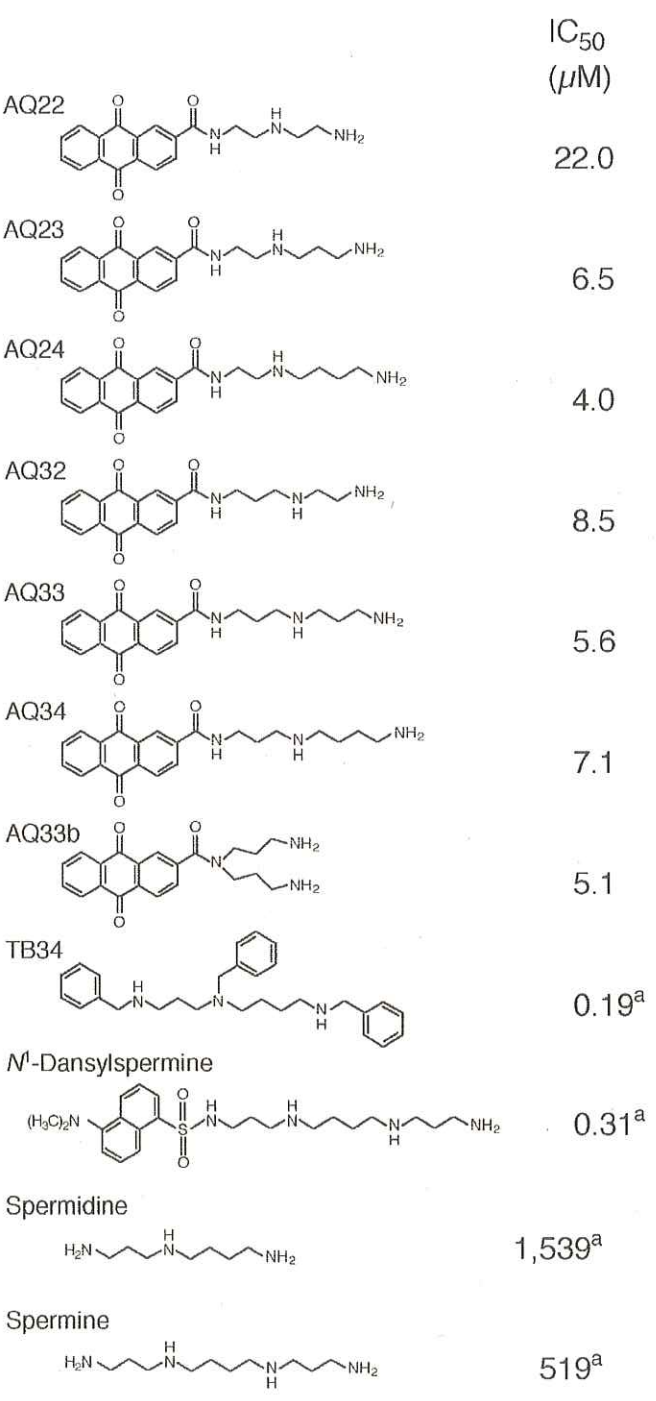

Fig. 1. Structures and activity of AQP derivatives and other polyamines. IC_{50} values were determined from concentration-inhibition curves ($n = 4$ oocytes) at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV. [a]Data for spermine, spermidine, N^1-dansylspermine, and TB34 are from Chao et al., 1997 and Igarashi et al., 1997.

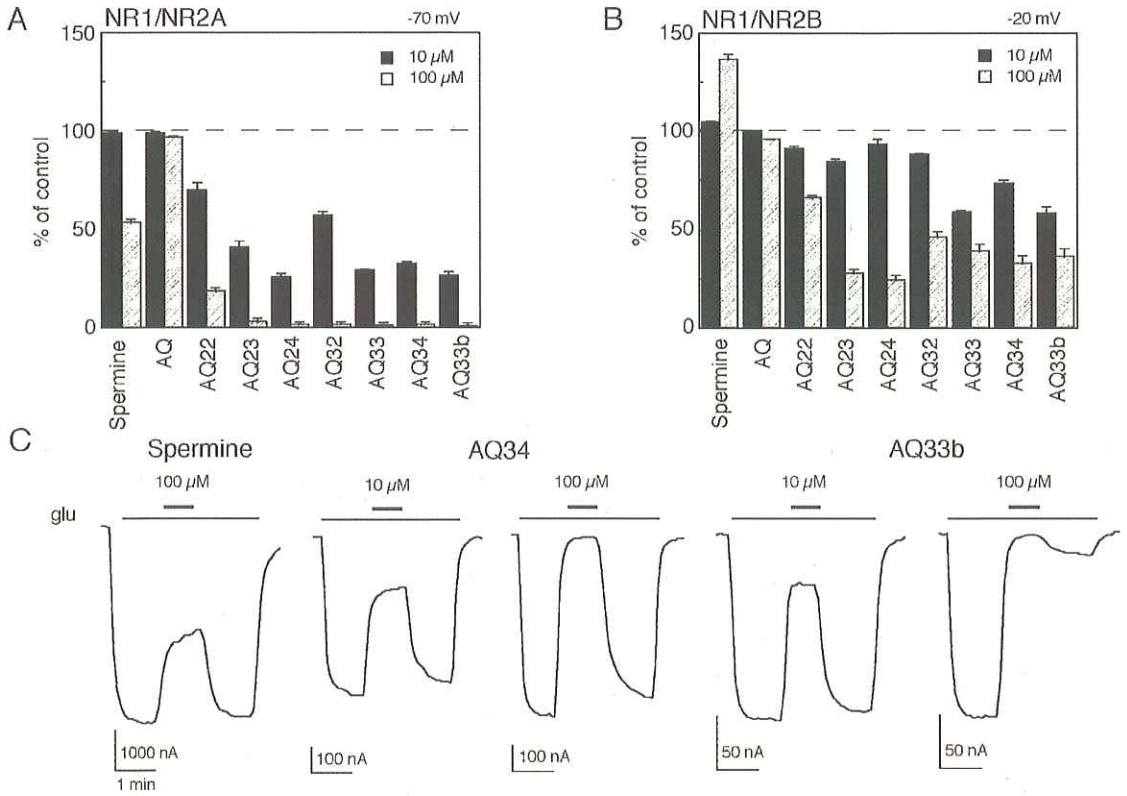

Fig. 2. Effects of AQP derivatives at NMDA receptors. The effects of AQP derivatives, each at 10 and 100 μM, on responses to glutamate (10 μM, with 10 μM glycine) were determined in oocytes expressing NR1/NR2A receptors voltage-clamped at −70 mV (A) and NR1/NR2B receptors voltage-clamped at −20 mV (B). Data are expressed as a percentage of the control response. Values are mean ± S.E.M. from four oocytes for each compound. C, representative traces showing block by spermine, AQ34, and AQ33b at NR1/NR2A receptors (Glu = 10 glutamate + 10 μM glycine). Recovery from block by 100 μM AQ33b was incomplete.

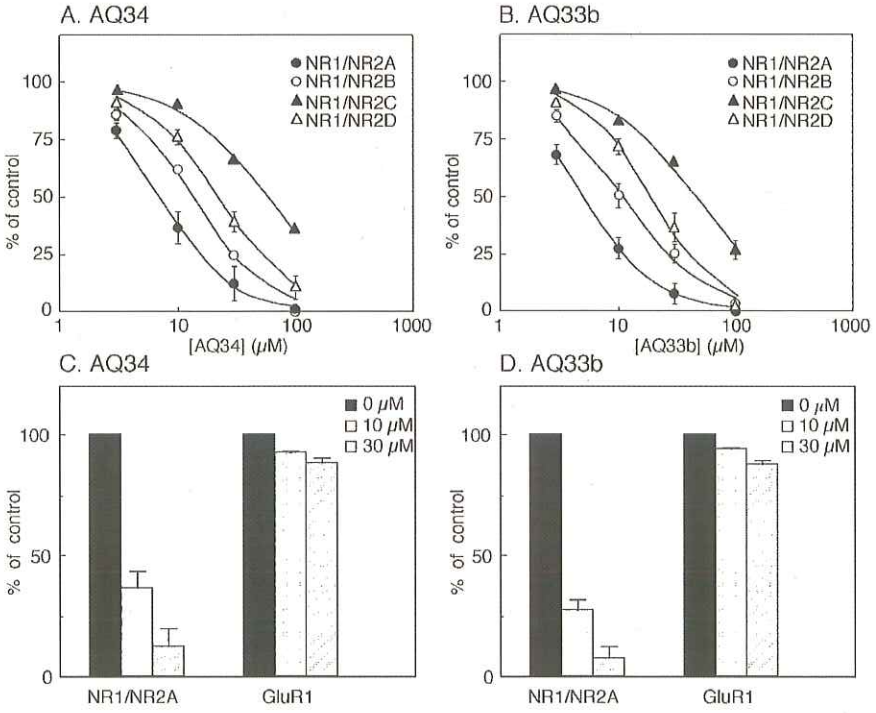

Fig. 3. Effects of AQP derivatives at NMDA and AMPA receptors. A and B, concentration-inhibition curves for AQ34 (A) and AQ33b (B) were determined at NR1/NR2 receptors containing NR2A, NR2B, NR2C, and NR2D. Values are mean ± S.E.M. from four oocytes for each subunit combination. C and D, effects of AQ34 (C) and AQ33b (D) were determined at NR1/NR2A receptors activated by glutamate (Glu, 10 μM, with 10 μM glycine) and at GluR1 receptors activated by 100 μM kainate in oocytes voltage-clamped at −70 mV. Responses to glutamate or kainate measured in the presence of AQ34 or AQ33b are expressed as a percentage of the control response at each receptor type. Values are mean ± S.E.M. from four oocytes.

of the membrane electric field sensed by the blocker at its binding site within that field, and F, R, and T have their usual meanings. In the fitting procedure, the parameters α, $K_d(0)$, and $z\delta$ were free. The α function was included in eq. 2 because, in some cells, the glutamate response showed a small run-down or run-up over time. The inclusion of the α variable improves the fitting procedure.

Synthesis of Anthraquinone Polyamines. The polyamine derivatives used in this study are shown in Fig. 1. AQ22 and AQ32 were synthesized from anthraquinone-2-carboxyl chloride (AQCC) according to the method of Okawara et al. (1990). AQ23 and AQ24 were prepared by treatment of protected triamines (*N*-[2-benzyloxy carbonylamino]ethyl)-*N,N'*-bis(*tert*-butoxycarbonyl)-diamine) with AQCC, and the Boc (*tert*-butoxycarbonyl) group was removed. AQ33b was prepared through phthalation of triamine followed by reaction with AQCC, and the phthaloyl group was removed. AQ33 and AQ34 were prepared from 1-(anthraquinone-2-carboxyl)amino-3-bromopropane and diamine. The structures of each compound were determined by spectral data and elemental analysis.

Results

Activities of AQP Derivatives. Polyamines, such as spermine, have various effects on NMDA receptors including stimulation and voltage-dependent block, which are dependent on the subunit composition of the receptors (Williams et al., 1994; Zhang et al., 1994). The various effects can be studied in relative isolation by the use of particular subunit combinations, agonist concentrations, and holding potentials. NR1/NR2B, but not NR1/NR2A, receptors are potentiated by spermine in the presence of saturating concentrations of agonist, whereas both subtypes are blocked in a voltage-dependent manner by spermine (Williams, 1994; Williams et al., 1994). Thus, to look for voltage-dependent block, AQP derivatives were studied at NR1/NR2A receptors in oocytes voltage-clamped at −70 mV. To look for stimulation, we measured effects at NR1/NR2B receptors voltage-clamped at −20 mV. At a concentration of 10 μM, all AQP derivatives inhibited responses to glutamate at NR1/NR2A receptors, and strong inhibition was observed with AQ23, AQ24, AQ33, AQ34, and AQ33b (Fig. 2A). By itself, the AQP head group AQ-2 sulfonic acid (AQ), was inactive indicating that the polyamine tail is essential for inhibitory activity. At NR1/NR2B receptors, the AQP derivatives showed inhibition but not potentiation of responses to glutamate (Fig. 2B). It is possible that potentiation by these analogs is masked by a potent, voltage-dependent channel block that is manifest even at −20 mV, but results of studies using voltage ramps (see below) suggests that this is not the case because stimulation was not seen even at depolarized potentials. Thus, unlike spermidine and spermine, AQP derivatives do not potentiate NR1/NR2B receptors. The AQP derivatives had IC_{50} values of 4 to 22 μM at NR1/NR2A receptors (Fig. 1).

The effects of two AQP derivatives, AQ34 and AQ33b, were characterized in detail. To determine the subunit selectivity of block by AQ34 and AQ33b at NMDA receptors, we measured concentration-inhibition curves at NR1/NR2 receptors containing the NR2A, NR2B, NR2C, and NR2D subunits (Fig. 3, A and B). For both AQ34 and AQ33b, there were significant differences in sensitivity between receptors containing various NR2 subunits. For both compounds, the largest difference (8- to 9-fold) was between NR1/NR2A and NR1/NR2C. To determine whether AQ34 and AQ33b were selective NMDA receptor antagonists, we studied their effects at AMPA receptors expressed from the GluR1 subunit and activated by the nondesensitizing agonist kainate (Fig. 3, C and D). At concentrations of 10 to 30 μM, which produce substantial inhibition at NMDA receptors, AQ34 and AQ33b had little effect at GluR1 receptors. Similar effects were seen at homomeric GluR2(Q) receptors, at which 10 μM AQ34 inhibited responses by 13 ± 1% (n = 9). Thus, the AQP derivatives preferentially block NMDA receptors.

Identification of Amino Acid Residues Involved in Block by AQP Derivatives. We carried out experiments to identify amino acid residues that are involved in the block of NMDA channels by AQ34 and AQ33b using a series of NR1 and NR2B mutants that we have previously generated to study the pore and vestibule structure in NMDA receptors

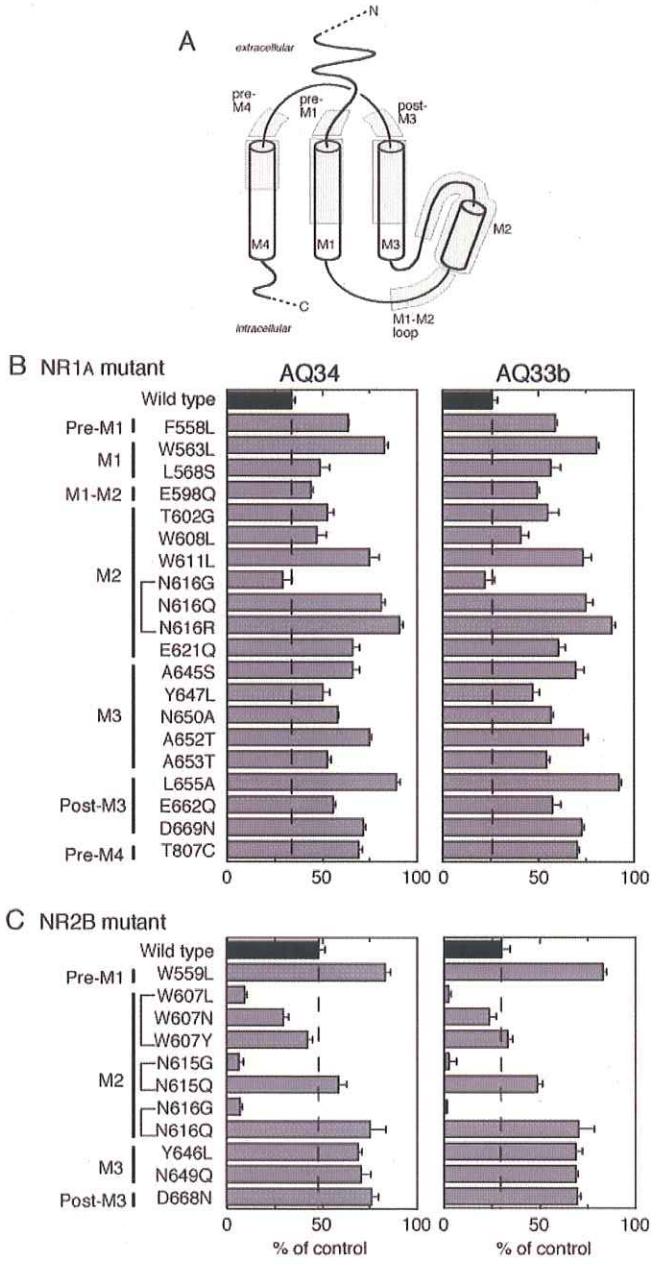

Fig. 4. Effects of AQ34 and AQ33b at receptors containing NR1 and NR2B mutants. A, schematic of the NR1 subunit, which contains three transmembrane segments (M1, M3, and M4), a re-entrant loop (M2), and a large extracellular loop between M3 and M4. The stippled boxes indicate the region in which mutations were studied. B, the effects of AQ34 and AQ33b (10 μM) were determined in oocytes expressing wild-type and mutant NR1/NR2A receptors and voltage-clamped at −70 mV. C, the effects of AQ34 and AQ33b at 10 μM were determined in oocytes expressing wild-type and mutant NR1/NR2B receptors and voltage-clamped at −70 mV. Values are mean ± S.E.M. from four oocytes for each mutant.

(Kashiwagi et al., 1997, 2002; Williams et al., 1998). We recently identified residues that differentially affect block by memantine, MK-801, and the polyamine derivative TB34 (Kashiwagi et al., 2002). TB34 (Fig. 1) is about 25-fold more potent than AQ34 and AQ33b at NR1/NR2A receptors (Igarashi et al., 1997), more hydrophobic than the parent compound spermidine, and still a highly flexible molecule but lacks a free polyamine "tail" as found in the AQP derivatives. Thus, the effects of 10 μM AQ34 and AQ33b were measured using NR1 and NR2B mutants which affect block by spermine, TB34, and NR1 mutants in the inner vestibule below the level of selectivity filter (Fig. 4). Of the amino acid residues tested, most mutations which reduced block by TB34 (Kashiwagi et al., 1997, 2002) also reduced block by AQ34 and AQ33b; however, mutations at a number of additional residues (NR1, Leu568, Glu598, Thr602, Trp608, and Trp611) reduced block by the AQP derivatives but not by TB34 (Kashiwagi et al., 2002). These amino acid residues are mainly located in the inner vestibule below the level of selectivity filter. The differential effects of these mutants on block by TB34 and AQP may be due to differences in the size and hydrophobicity of the anthraquinone and benzene substitutions of these two classes of polyamines and/or to the presence of a "free" polyamine tail with unsubstituted amino groups in the AQP derivatives.

Residues at the Selectivity Filter. Mutations at the critical asparagine in the M2 loop of NR1 (N616Q and N616R) reduced block by AQ34 and AQ33b (Fig. 4); however, substitution of Gly for Asn slightly enhanced the block by AQ34 and AQ33b. Similar results were obtained with N^1-dansylspermine, which has an intact polyamine tail with a hydrophobic head group (Chao et al., 1997). 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 sites (Dingledine et al., 1999). Substitution of Gln for Asn at either position reduced block by AQ34 and AQ33b, whereas substitution of Gly for Asn increased block (Fig. 4). A tryptophan residue in the M2 loop of NR2B (Trp607) has also been proposed to contribute directly to the selectivity filter or to markedly influence the structure and position of the selectivity filter and the binding site for extracellular Mg^{2+} (Williams et al., 1998). Mutations at NR2B(Trp607) to Leu or Asn also increased block by AQ34 and AQ33b (Fig. 4C).

When mutations were made at NR1(Asn616), NR2B-(Trp607), NR2B(Asn615), and NR2B(Asn616) to reduce the size of the side chain (Asn to Gly or Trp to Leu), in all cases sensitivity to AQ34 increased (Fig. 5). The sensitivity of a number of double mutants was examined by coexpressing NR1(N616G) with each of several NR2B mutants (Fig. 5D). The sensitivity to AQ34 was nearly equal in the three double mutants, and it was slightly higher than that of the single mutants NR2B(W607L) and NR2B(N616G). The sensitivity of the double NR1(N616G)/NR2B(N615G) mutant was less than that of the single NR2B(N615G) mutant, possibly due to increased permeation of AQ34 through the channel in the double mutant (see below). Similar results were seen with mutations at corresponding positions in NR2A, although the effect of the NR2A(W606L) mutant was less than that of the corresponding NR2B(W607L) mutant (Fig. 5, C and E).

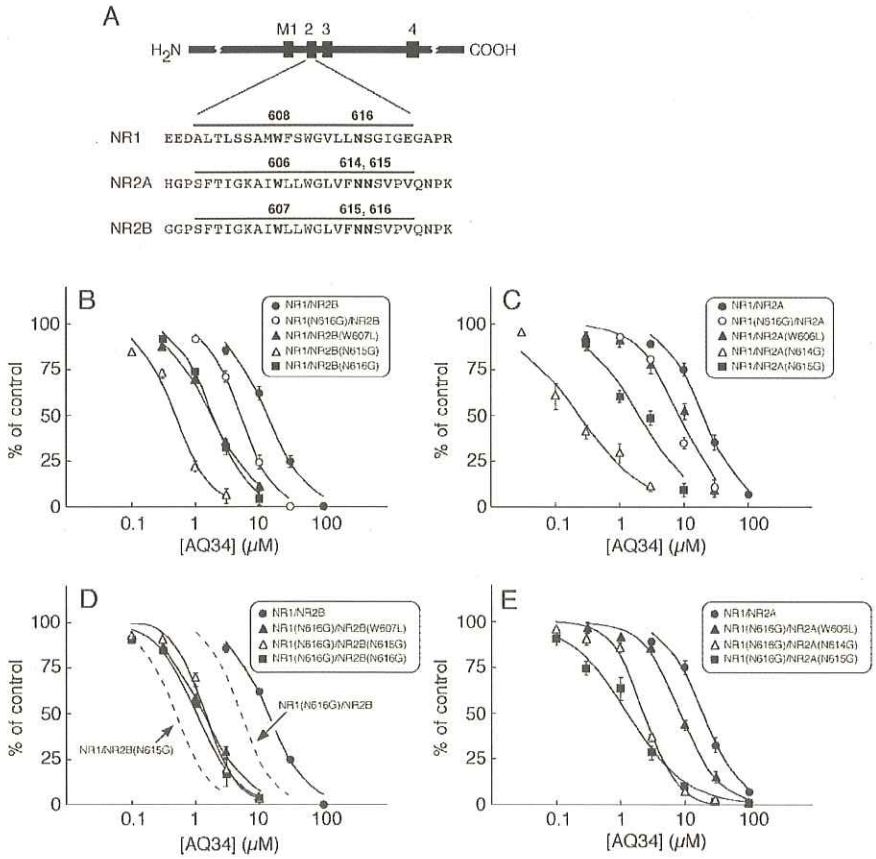

Fig. 5. Effects of AQ34 on NMDA receptors containing mutations at the selectivity filter. The positions of the M1-M4 segments of NMDA receptor and amino acid sequences of the M2 region of NR1, NR2A, and NR2B are shown in panel A. Concentration-inhibition curves for AQ34 were determined at NR1/NR2B (B and D) receptors and NR1/NR2A receptors (C and E) containing single or double mutations and voltage-clamped at −70 mV. Values are mean ± S.E.M. from four oocytes for each mutant.

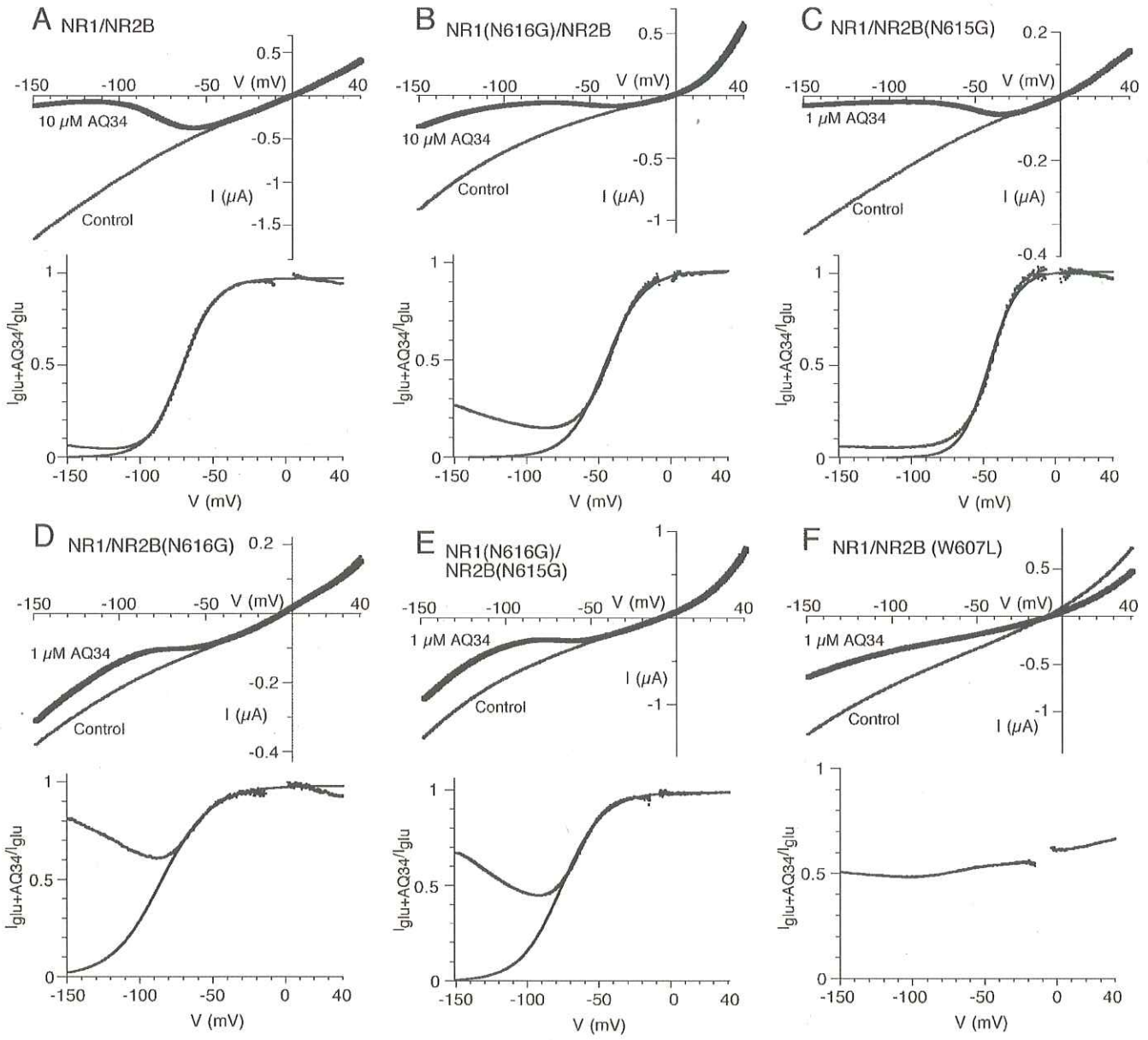

Fig. 6. Block and permeation of AQ34 at mutant NMDA channels. Top panels, I-V curves were constructed by voltage ramps from −150 to +40 mV in oocytes expressing wild-type and mutant receptors. Responses to 10 μM glutamate (with 10 μM glycine) were measured in the absence (control) and presence of AQ34. Leak currents were subtracted. Bottom panels, currents measured in the presence of AQ34 ($I_{Glu+AQ34}$) are expressed as a fraction of the control current (I_{Glu}) for each oocyte. Data around the reversal potential have been masked. The solid lines are fits to the Woodhull model (eq. 2). Note the incomplete block at some mutant NMDA receptors and the apparent reversal of block and restoration of inward current at very negative potentials. At NR1/NR2B(W607L) (F), block by 1 μM AQ34 showed no apparent voltage dependence and was not relieved even at positive membrane potentials. Similar effects were seen with 0.3 to 10 μM AQ34 at NR1/NR2B(W607L) receptors (data not shown).

Block of NMDA channels by spermine and by a number of polyamine analogs is voltage-dependent, but the degree of voltage dependence and/or the depth of the binding site varies between different polyamine analogs. For example, N^1-dansylspermine ($z\delta$ = 2.58; assuming z = 4 then δ = 0.65) shows a stronger voltage dependence than TB34 ($z\delta$ = 1.38; assuming z = 3 then δ = 0.46) (Chao et al., 1997; Kashiwagi et al., 2002). Some mutations at the selectivity filter have been shown to alter the apparent permeation of polyamines and polyamine derivatives. Thus, we carried out experiments to study the voltage dependence and permeation of AQ34 and the effects of mutations at the selectivity filter on block and permeation. The purpose of these experiments was 2-fold: first, to study voltage dependence using the Woodhull (1973) model of voltage-dependent block to determine values for the $K_d(0)$ and for the apparent valence, $z\delta$; and second, to determine whether AQ34 shows significant permeation of the channel manifest as a partial relief of block and restoration of an inward current at very negative membrane potentials. As an index of the degree of permeation, we calculated the ratio of the fractional current in the presence of AQ34 at −130 mV compared with the fractional current in the presence of AQ at −70 mV ($F_{-130/-70}$). These two voltages were chosen arbitrarily but are within the range where AQ34 produces a

TABLE 1

Effects of NR1 and NR2 mutants on block by AQ34

Values for $K_d(0)$ and $z\delta$ were determined using voltage ramps analyzed with the Woodhull model of voltage-dependent block. $F_{-130/-70}$ is the ratio of the fractional current in the presence of AQ34 at −130 and −70 mV. Data are the mean ± S.E.M.

Receptor	$K_d(0)$	$z\delta$	$F_{-130/-70}$	n
	μM			
Wild-type NR/NR2B	3481 ± 205	2.12 ± 0.02	0.10 ± 0.01	29
NR1(N616G)/NR2B	344 ± 29	2.00 ± 0.05	1.23 ± 0.05	4
NR1/NR2B(N615G)	216 ± 40	2.77 ± 0.05	0.30 ± 0.15	12
NR1/NR2B(N616G)	190 ± 39	1.75 ± 0.09	1.31 ± 0.10	10
NR1(N616G)/NR2B(N615G)	345 ± 172	2.12 ± 0.16	0.80 ± 0.11	4
NR1(N616G)/NR2B(N616G)	9 ± 24	2.09 ± 0.11	1.62 ± 0.04	7
NR1/NR2B(W607L)			1.00 ± 0.01	11
NR1/NR2B(W610L)	4381 ± 331	2.21 ± 0.05	0.14 ± 0.01	5
Wild-type NR1/NR2A	5450 ± 1482	2.31 ± 0.05	0.08 ± 0.02	13
NR1/NR2A(N614G)	180 ± 42	2.62 ± 0.10	0.12 ± 0.04	7
NR1/NR2A(N615G)	142 ± 44	1.83 ± 0.07	1.11 ± 0.08	12
NR1(N616G)/NR2A(N614G)	353 ± 89	1.97 ± 0.11	0.92 ± 0.04	5
NR1(N616G)/NR2A(N615G)	169 ± 22	2.24 ± 0.08	1.59 ± 0.05	8
NR1/NR2A(W606L)	672 ± 237	1.68 ± 0.09	0.86 ± 0.02	5
NR1/NR2A(W609L)	4362 ± 797	2.21 ± 0.05	0.13 ± 0.04	6

n, number of oocytes used.

strong block at wild-type receptors. At wild-type receptors, for a strongly voltage-dependent blocker that does not permeate the channel, there will be a much greater block at −130 compared with −70 mV. Thus, the value of $F_{-130/-70}$ will be much smaller than 1.0. If the blocker shows significant permeation with a particular mutant, there will be less of an increase in the degree of block between −130 and −70 mV, and the value of $F_{-130/-70}$ will be greater than the wild-type and will approach 1.0. When there is dramatic permeation, there may actually be less block at −130 than at −70 mV, and the value of $F_{-130/-70}$ will be greater than 1.0. An increase in permeation may reflect an increase in the size of the narrow constriction at the selectivity filter.

At wild-type NR1/NR2B and NR1/NR2A receptors, block by AQ34 was strongly voltage-dependent with a $z\delta$ value of 2.1 to 2.3 and a $K_d(0)$ value of 3.4 to 5.4 mM (Fig. 6A; Table 1). Block of wild-type receptors was almost complete with little permeation ($F_{-130/-70}$ = 0.08–0.10) at very negative membrane potentials. There was a small increase in permeation with Asn to Gly mutation at NR1(N616G) and a large increase in permeation with the NR2B(N616G) mutant (Fig. 6, B and D; Table 1). Enhanced permeation was also seen with the double mutations NR1(N616G)/NR2B(N615G) and NR1(N616G)/NR2B(N616G) (Table 1). The results suggest that NR1(Asn616) and NR2B(Asn616) are the key determinants of the narrowest part of the channel. Similar results were obtained with NR2A mutants. Thus, AQ34 could easily permeate NR1/NR2A(N615G), NR1(N616G)/NR2A(N614G), and NR1(N616G)/NR2A(N615G) but not NR1/NR2A(N614G) (Table 1). The NR1 and NR2 mutations primarily affected the $K_d(0)$ suggesting an increased affinity of binding although, in some cases, there were changes in $z\delta$ (Table 1). This could reflect a shift in the depth of the binding site due to the mutation, but the incomplete block may also distort the Woodhull (1973) fit, as we could only fit the initial component of block in mutants where there was considerable breakthrough at very negative potentials (see lower panels in Fig. 6, D and E).

The NR2B(W607L) mutant increased the potency of AQ34 (Fig. 5B); however, block of NR1/NR2B(W607L) receptors by AQ34 showed no apparent voltage dependence (Fig. 6F). This is a surprising result, and it may suggest that NR2B(Trp607) is important for maintenance of the structure of the channel.

Discussion

Glutamate receptor-mediated neurotoxicity contributes to a variety of neurological disorders as well as cell death following trauma and stroke. Development of clinically useful compounds is therefore important, especially given that few glutamate receptor blockers simultaneously satisfy the requirements of effectiveness and safety or lack of side effects. In this regard, 2,3-benzodiazepines and amino-alkyl-cyclohexanes have been recently developed as glutamate receptor antagonists (Rogawski, 1993; Parsons et al., 1999). Polyamine derivatives are also potential lead compounds for novel, therapeutically useful NMDA antagonists and are useful as tools to study NMDA and other glutamate receptors. In this study, we have found that AQP derivatives preferentially block NMDA receptors. The AQP derivatives are all triamines and were 70- to 400-fold more potent than the natural triamine spermidine but about 10-fold less potent than N^1-dansylspermine and TB34. N^1-Dansylspermine blocks NMDA channels and is also a potent and poorly reversible antagonist at AMPA [GluR1 or GluR2(Q)] and δ2 glutamate receptors (Williams et al., 2003) whereas TB34, like the AQP derivatives, is a selective NMDA receptor blocker (Igarashi et al., 1997). This indicates that the nature of the head group on these relatively simple polyamine derivatives is an important determinant of channel selectivity, presumably reflecting differences in the interaction with different residues in NMDA and AMPA channels or differences in the accessibility of the binding sites within these channels.

We have recently constructed a model of the NMDA channel pore and vestibule regions (Kashiwagi et al., 2002) based on the results of mutagenesis studies and results from previous studies in which solvent-accessible residues were probed after cysteine substitution (Kuner et al., 1996; Beck et al., 1999) and crystallographic studies describing the structure of a bacterial K^+ channel (Doyle et al., 1998). We have used this model to map the amino acid residues at which mutations influence block by AQP and to compare those

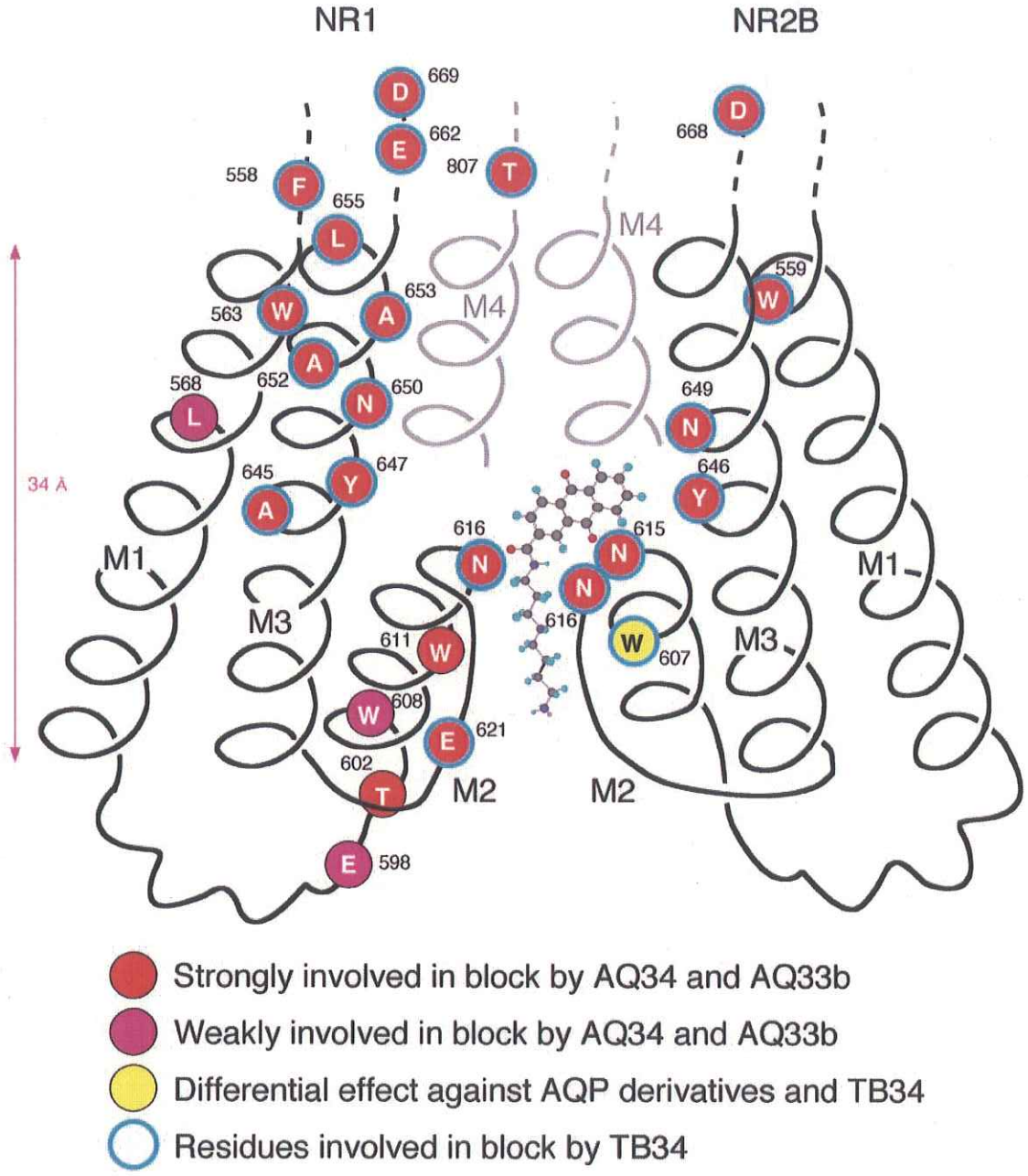

Fig. 7. Modeling residues that affect block by AQ34 and AQ33b in the pore and vestibule of the NMDA receptor. The M1-M2-M3 region is depicted as a helix-pore-loop helix similar to the structure reported for the KcsA potassium channel from *Streptomyces lividans* (Doyle et al., 1998). The M2 region contains a helix followed by a random coil structure. Red circles indicate residues at which mutations strongly affect block by AQ34 and AQ33b. Pink circles indicate residues at which mutations weakly affect block by AQ34 and AQ33b. The yellow circle indicates a residue at which mutations have differential effects on block by AQP derivatives and TB34. Circles outlined in light blue indicate residues at which mutations strongly affect block by TB34 (Kashiwagi et al., 2002).

residues with mutations that affect block by TB34 (Fig. 7). Residues that influence AQ34 and AQ33b are located mainly on the M2 loop and the M3 transmembrane segment, similar to residues that affect block by TB34; however, some residues that affect block by AQP derivatives did not influence TB34. These residues include NR1, Leu568, Glu598, Thr602, Trp608, and Trp611 (Fig. 7). Notably, four of these residues are located below the level of the selectivity filter. It is conceivable that the polyamine tail in AQ34 normally passes through the filter and interacts with these residues, even though the head group cannot easily permeate the narrow constriction (Fig. 7). In contrast, TB34 is too bulky to even partially pass through the narrow constriction in wild-type channels (Kashiwagi et al., 2002). This would also be consistent with the idea that the binding site for AQ34 ($z\delta = 2.12$; assuming $z = 3$ then $\delta = 0.71$) is deeper in the channel pore than is the site for TB34 ($z\delta = 1.38$; assuming $z = 3$ then $\delta = 0.46$). Consistent with this idea, there is a report that the adamantane derivatives IEM-1754 and IEM-1857, which are NMDA receptor channel blockers and have an amine tail,

have a binding site below the level of the selectivity filter (Antonov et al., 1998). It has been also reported that a number of residues in the intracellular vestibule of non-NMDA receptors are involved in the binding of polyamines (Panchenko et al., 1999; 2001). It also remains possible that these mutations in these regions nonspecifically disrupt the structure of the channel. Indeed, it may be that the structure of the channel is changed by substitution of Leu for Trp at NR2B(Trp607) as discussed below.

If the binding site for AQ34 lies deep within the channel near the narrow constriction (Fig. 7), then why do mutations at more peripheral residues in M1 and M3 (e.g., Asp669, Leu655, Trp563, etc. in NR1) affect block by AQ34? One possibility is that these residues affect the accessibility of AQ34 for its binding site rather than contributing to the binding site per se. That is, these residues may be involved in gating of the channel or may control access of the blocker as it passes into the channel. Consistent with this idea, many of the peripheral residues in the channel mouth that affect block by AQP derivatives also affect block by MK-801, a relatively small and rigid molecule that probably binds deep within the pore near the selectivity filter (Kashiwagi et al., 2002). Portions of the external vestibule, in particular the M3 segment, appear to undergo significant movement during gating, and the relative positions of these segments are staggered between NR1 and NR2 subunits (Sobolevsky et al., 2002a,b). A staggering of these regions of the external vestibule may account for differences in the effects of mutations at homologous positions in NR1 and NR2 subunits, particularly if these regions affect the accessibility of channel blockers to their binding site near the selectivity filter. Our data may also be consistent with the proposal that a second external binding site for NMDA receptor channel blockers exists in addition to a deep site near the selectivity filter (Subramaniam et al., 1996; Sobolevsky and Koshelev, 1998; Bolshakov et al., 2003).

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 NR2B(Asn615) also contributes to the selectivity filter (Kuner et al., 1996; Wollmuth et al., 1996; Dingledine et al., 1999). A similar relationship was observed for the corresponding residues (Asn614 and Asn615) in NR2A. The results from studies using voltage ramps suggest that NR2B(Trp607) also plays an important role to maintain the structure of channel pore and narrow constriction. This residue has previously been shown to have a profound influence on block and permeation of TB34, MK-801, Mg^{2+}, and several other blockers (Williams et al., 1998; Kashiwagi et al., 2002). Notably, the effects of mutations at NR2B(Trp607) were markedly different for AQ34 compared with TB34. For example, with NR2B(W607L) the equilibrium block by AQ34 is increased (the IC_{50} is decreased), whereas the equilibrium block by TB34 is greatly reduced (Kashiwagi et al., 2002). Block by AQ34 shows little voltage dependence in receptors containing the NR2B(W607L) mutant. It may be that the blocker accumulates near the intracellular mouth of the channel but does not easily pass back through the channel. Thus, mutations at NR2B(Trp607) may eliminate the deep binding site for AQ34 leaving only a voltage-independent binding site near the intracellular or extracellular mouth of the channel. There is a tryptophan, Trp606, at the analogous position in NR2A, but an NR2A(W606L) mutation had more modest effects than the corresponding NR2B(W607L) mutation. There may be differences in the positions of these Trp residues in NR2A and NR2B relative to the critical Asn residues in the M2 loop, which could explain the more drastic effects of mutations in NR2B as compared with NR2A. In this context, the NR2B(W607L) mutation was previously shown to have a much greater effect on block and permeation of Mg^{2+} than the corresponding NR2A(W606L) mutation (Williams et al., 1998).

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