Pergamon

Neuropharmacology 39 (2000) 1681–1691

NEURO PHARMACOLOGY

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Alcohols inhibit *N*-methyl-D-aspartate receptors via a site exposed to the extracellular environment

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Accepted 21 March 2000

Abstract

N-Methyl-D-aspartate (NMDA) receptors are important CNS target sites of alcohols, but the site and mechanism of action of alcohols on NMDA receptors remains unclear. In CHO-K1 cells transfected with NR1/NR2B NMDA receptor subunits, ethanol inhibited NMDA-activated current with an IC_{50} of 138 mM. Truncation of the intracellular C-terminal domain of the NR1 subunit (NR1T) did not alter ethanol sensitivity when combined with the NR2B subunit, but a similar truncation of the NR2B subunit (NR2BT) slightly enhanced ethanol sensitivity of receptors formed from coexpression with either NR1 or NR1T subunits. 1-Pentanol applied externally inhibited NMDA receptors with an IC_{50} of 9.9 mM, but intracellular application of 1-pentanol (25 mM) did not alter NMDA receptor inhibition by externally applied ethanol or 1-pentanol. In addition, the amplitude of NMDA-activated current did not decrease during the time required for 1-pentanol (25 mM) to diffuse throughout the cytoplasm. Ethanol did not inhibit NMDA receptors when bath-applied in cell-attached patches or when applied to the cytoplasmic face of inside-out membrane patches. These results appear to be best explained by an action of alcohols on the NMDA receptor-channel protein, at a site located in a domain exposed to, or only accessible from, the extracellular environment. Published by Elsevier Science Ltd.

Keywords: NMDA; Alcohol; Ethanol; Pentanol; Ion channel; Extracellular

1. Introduction

The biological actions by which alcohols such as ethanol produce their well-known behavioral effects have not been established. Alcohols undoubtedly interact with many sites in the central nervous system, and most probably produce their biological effects through combined actions at a number of these sites. Of the proposed targets of alcohol action, however, accumulating evidence implicates neurotransmitter-gated membrane ion channels as among the most important (Diamond and Gordon, 1997). The *N*-methyl-D-aspartate (NMDA) receptor-ion channel, a subtype of the glutamate-gated membrane ion channel that is involved in nervous system excitability, cognitive function, and motor coordination (Morris et al., 1986; Willetts et al., 1990; Bliss and Collingridge, 1993), is thought to play an important role in mediating the intoxicating effects of alcohols. Alcohols inhibit NMDA receptor function at pharmacological concentrations (Göthert and Fink, 1989; Hoffman et al., 1989; Lovinger et al. 1989, 1990), alcohols and NMDA receptor antagonists can produce similar subjective effects in animals and humans (Grant et al., 1991; Hodge and Cox, 1998; Krystal et al., 1998), and the potency of alcohols in producing behavioral effects is similar to, and correlated with, their NMDA receptor inhibitory potency (Lovinger et al., 1989; Peoples and Weight 1995, 1999).

The molecular mechanism by which alcohols modulate NMDA receptor function, however, remains unclear. Alcohols are known to inhibit NMDA receptors in a manner that is noncompetitive with respect to the agonist (Göthert and Fink, 1989; Gonzales and Woodward, 1990; Rabe and Tabakoff, 1990; Peoples et al., 1997). Recent studies have not identified interactions of alcohols with a number of the modulatory sites of the NMDA receptor-channel (Chu et al., 1995; Peoples et al., 1997),

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0028-3908/00/$ - see front matter. Published by Elsevier Science Ltd.
PII: S0028-3908(00)00067-8

and alcohols do not appear to act by binding within the ion channel pore (Wright et al., 1996). Although a number of studies have reported an interaction of alcohols with the glycine coagonist site (Hoffman et al., 1989; Rabe and Tabakoff, 1990; Woodward and Gonzales, 1990; Dildy-Mayfield and Leslie, 1991; Buller et al., 1995), other studies have not observed such an interaction (Gonzales and Woodward, 1990; Peoples and Weight, 1992; Woodward, 1994; Chu et al., 1995; Mirshahi and Woodward, 1995; Cebers et al., 1996; Peoples et al., 1997).

The inability to demonstrate an interaction of alcohols with a known site on the NMDA receptor-channel protein could be interpreted as evidence that such a site does not exist, and that alcohols affect NMDA receptor function via effects on membrane lipids, or on one or more proteins that interact with the NMDA receptor, such as protein kinase C (Snell et al., 1994). Although the observation that alcohol inhibition of NMDA receptors exhibits a "cutoff" effect (loss or plateau in potency of a series of alcohols when the alcohol carbon chain length exceeds a certain point) is consistent with a direct action of alcohols on NMDA receptors (Peoples and Weight, 1995), recent results suggest that the cutoff phenomenon in NMDA receptors does not result purely from a size exclusion mechanism (Peoples, 1999), as was originally proposed. Similarly, the observation that ethanol inhibition of NMDA receptor-mediated single-channel current is preserved in excised outside-out membrane patches is also consistent with a direct action of ethanol on the NMDA receptor, but this could also result from an effect of ethanol on other proteins closely associated with the NMDA receptor that were present in the membrane patches. The results of the present study thus constitute the clearest evidence obtained to date that alcohols inhibit the NMDA receptor via a direct interaction with the receptor protein, and also suggest that the site of alcohol action on this receptor is located either on an extracellular domain or on a region of the receptor that is accessible only from the extracellular environment. Part of this work has appeared previously in abstract form (Peoples, 1998; Peoples and Stewart, 1999).

2. Methods

2.1. Cell culture

Chinese hamster ovary (CHO) K1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in a medium consisting of Ham's F-12K nutrient mixture containing 2 mM L-glutamine and 1.5 g/l sodium bicarbonate, 90%, and fetal bovine serum, 10%.

2.2. Transient transfection

NMDA receptor cDNA clones for the rat NR1-1a and NR2B subunits were gifts from Drs D.R. Lynch (University of Pennsylvania) and D.M. Lovinger (Vanderbilt University). CHO cells were seeded in 35-mm dishes and allowed to grow to 70–95% confluence, and were transfected with cDNA for the NR1 and NR2B subunits and green fluorescent protein (pGreen Lantern; Life Technologies, Inc., Rockville, MD) in a 2:2:1 ratio, respectively, using LipofectAMINE PLUS or LipofectAMINE 2000 (Life Technologies). The culture medium during and after the transfection step contained 100 μM ketamine and 200 μM D,L-2-amino-5-phosphonovaleric acid (APV) to minimize cell death due to excitotoxicity. Cells were mechanically dissociated 18–48 h after transfection, and were replated at low density (~10–30% confluence) on polyornithine-coated 35-mm dishes at least 1 h prior to recording. This procedure resulted in a large number of green fluorescent protein-positive cells that were roughly spherical in shape, ~20 μm in diameter, and not in physical contact with other cells.

2.3. Construction of C-terminal truncation mutant NMDA receptor subunits

The NR1 C-terminal truncation mutant was made by polymerase chain reaction (PCR) using primer sets designed such that the upper primer (CCG GCA TTG GGG AAG GTG) annealed 5′ to the BspEI site of NR1 and the lower primer (TAT CTA GA*T GTA CA***T CA**G GCG ATC TCA ATG AAA ATG AGG A) annealed just 3′ to the transmembrane region #4 of NR1. The lower primer also introduced an in-frame termination codon shown in bold (**TGA** sense) and a BsrGI site shown in italics (*TGTACA* sense). The sequence of the C-terminal region of clone 14 (NR1T) was determined on an ABI-310 sequencing machine by automatic cycle sequencing with D-rhodamine dye terminators. The sequencing revealed that the termination codon and BsrGI sites were correct and in the appropriate locations. A map of the NR1 C-terminal truncation mutant is shown in Fig. 1A. Wild-type and truncated forms of the NR1 subunit were digested with Bsp EI and Not I as shown in Fig. 1C (see lanes NR1 wt and NR1T) to demonstrate that the truncated NR1 subunit had been produced.

The NR2B C-terminal truncation mutant was also made by PCR using primer sets designed so the upper primer (AGG GTG GGG AAA TGG AAG GAC AAG) annealed 5′ to the Afl II site of NR2B and the lower primer (TT*G ATA TC***T CA**A TGC CGG AAC TGC CAA TAG AAC) annealed just 3′ to the transmembrane region #4 of NR2B. The lower primer also introduced an in-frame termination codon (**TGA** sense) and an EcoRV site (*GATATC* sense). Sequencing of the C-ter-

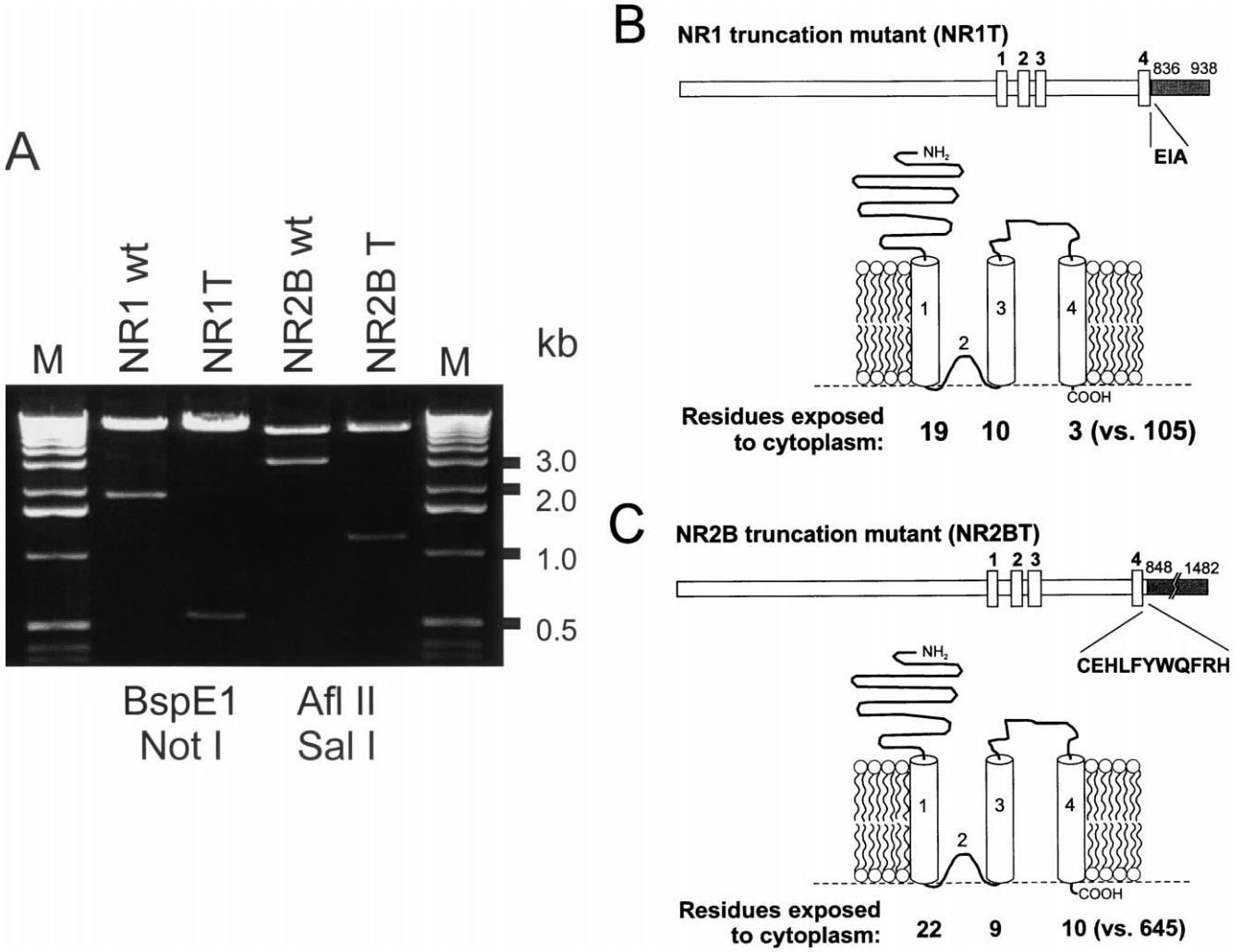

Fig. 1. C-terminal truncation mutants of the NMDA receptor NR1 subunit (NR1T) and NR2B subunit (NR2BT). (A) Restriction digests of plasmids containing wild-type NR1 (NR1 wt), NR1-T, wild-type NR2B (NR2B wt) and NR2BT to illustrate removal of sequences 3′ to the sequences coding for residues of the fourth transmembrane region. The expected fragment sizes of NR1 wt and NR1T, after digestion with BspE1 and NotI, were 7837 and 1837 bp for the wild-type plasmid and 7837 and 558 bp for the truncated plasmid. For NR2B wt and NR2BT plasmids, digestion with Afl II and Sal I yielded bands of 6488 and 3132 bp for the wild-type plasmid and 6488 and 1176 bp for the truncated construct. Thus, the DNA sequences coding for amino acids constituting most of the C-terminal domains of NR1 and NR2B subunits have been removed. Diagrams of the C-terminal truncation mutants of NR1 (B) and NR2B (C). Regions deleted from wild-type subunits are shown in dark shading, the positions of the C-terminal residues in the truncated and wild-type subunits are shown above the diagrams, and the remaining sequence following TM4 is shown below the diagrams. Note that the number of residues exposed to the intracellular environment was reduced from 134 to 32 in the NR1T subunit, and from 676 to 41 residues in the NR2BT subunit, compared to the respective wild-type subunits.

minal region of this clone (NR2BT) by cycle sequencing with ThermoSequenase and ^{33}P terminators (Amersham, Piscataway, NJ) revealed that the termination codon was in the correct location, but the EcoRV site was lost due to a single nucleotide deletion. A map of the NR2B C-terminal truncation mutant is shown in Fig. 1B. Finally, wild-type and truncated forms of the NR2B subunit were digested with Afl II and Sal I as shown in Fig. 1C (see lanes marked NR2B wt and NR2BT) to show that the C-terminus had been shortened.

2.4. Electrophysiological recording

Patch-clamp recording was performed at room temperature (22–25°C) using an Axopatch 200 or 200B (Axon Instruments Inc., Foster City, CA) patch-clamp amplifier. Gigaohm seals were formed using fire-polished patch-pipettes with tip resistances of 2–5 MΩ. Series resistances of 4–15 MΩ were compensated by 80–90%. Membrane potential was held at −50 mV in whole-cell recordings and +50 mV in cell-attached and inside-out patches. In most cases, data were filtered at 2

kHz (8-pole Bessel) and acquired on computer (5 kHz sampling frequency) during experiments using a DigiData 1200A interface and AxoTape or pCLAMP software (Axon Instruments). In single-channel experiments, data were filtered at 4 kHz (8-pole Bessel) and were recorded on videotape using a VR-10B digital data recorder (Instrutech Corp., Great Neck, NY) connected to a videocassette recorder (Sony SLV-440). Data were later replayed, filtered at 2 kHz (8-pole Bessel), and acquired at a sampling frequency of 10 kHz on a computer using a DigiData 1200A interface and pCLAMP software (Axon Instruments). Charge quantitation of single-channel data was performed using pCLAMP software; events were detected using a 50% threshold detection criterion.

2.5. Experimental solutions

Cells were superfused at 1–2 ml/min in an extracellular medium containing (in mM): NaCl, 150; KCl, 5; $CaCl_2$, 0.2; HEPES, 10; glucose, 10; pH was adjusted to 7.4 using NaOH and osmolality to 340 mmol/kg using sucrose. Low Ca^{2+} was used to minimize NMDA receptor inactivation (Zilberter et al., 1991). Unless noted otherwise, the patch-pipette solution in whole-cell recordings contained (in mM): CsCl, 95; Mg_4ATP, 4; 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 10; HEPES, 10; creatine phosphate, 20; creatine phosphokinase, 50 U/ml; pH was adjusted to 7.4 using CsOH and osmolality to 310 mmol/kg using sucrose. In cell-attached and inside-out patches, the patch-pipette solution consisted of extracellular medium containing NMDA, 100 μM, and glycine, 10 μM. Solutions of agonists and alcohols were prepared fresh daily in extracellular solution. Ethanol (95%, prepared from grain) was obtained from Pharmco (Brookfield, CT), 1-pentanol was obtained from Aldrich Chemical Co. (Milwaukee, WI), ketamine was obtained from RBI (Natick, MA), and all other drugs were obtained from Sigma Chemical Co. (St Louis, MO).

2.6. Intracellular perfusion

In some cases, an alcohol was added to the patch-pipette solution and sufficient time was allowed for the alcohol to diffuse throughout the cytoplasm. The time required for the alcohol concentration to equilibrate in the interior of the cell was determined by using the equation

$$\tau_d = 0.6 R_A (\mathrm{MW})^{1/3} (C_M/5.91)^{1.5} \quad (1)$$

where τ_d is the time constant for diffusion in seconds, R_A is access (series) resistance in MΩ, MW is the molecular weight of the diffusing substance (alcohol), and C_M is the cell capacitance in pF (Pusch and Neher, 1988). After rupturing the cell membrane within the patch-pipette to gain access to the cell interior, an interval of at least (3–5)×τ_d (i.e. 95–99% equilibration of the alcohol concentration in the cytoplasm) was allowed to elapse before the alcohol was considered to have perfused the interior of the cell.

2.7. Agonist and drug application

Solutions of agonists and drugs were applied to cells using a linear multi-barrel array (Peoples and Weight, 1995) in experiments using intracellular application of 1-pentanol, and a rapid solution exchange apparatus (Li et al., 1998) in the remaining experiments. Solutions containing excitatory amino acids were applied at intervals of at least 90 s, unless noted otherwise.

2.8. Curve fitting and statistical analysis

Concentration–response data were analyzed using the nonlinear curve-fitting program ALLFIT (DeLean et al., 1978), which uses an analysis of variance (ANOVA) procedure. Values reported for concentration yielding 50% of maximal inhibition (IC_{50}) and slope factor (n) are those obtained by fitting the data to the equation:

$$y = E_{max}/[1+(x/IC_{50})^n] \quad (2)$$

where x and y are concentration and response (e.g. percent inhibition), respectively, and E_{max} is the maximal response. Statistical evaluation of differences among means was determined by ANOVA or Student's t-tests using the program InStat (GraphPad Software, San Diego, CA). All values are reported as the mean±S.E.M.

2.9. Prediction of ethanol inhibition in the presence of intracellular 1-pentanol

Predicted values for ethanol inhibition of NMDA-activated current in the presence of intracellular 1-pentanol, assuming that extracellular and intracellular 1-pentanol were equipotent, and that 1-pentanol and ethanol acted at the same site, were calculated as follows. The percent inhibition of NMDA-activated current by 25 mM extracellular 1-pentanol was determined to be 80% (see Fig. 4B); the concentration of ethanol that produced this level of inhibition was 430 mM (see Fig. 5B). Thus if 25 mM 1-pentanol applied internally acted at the same site as ethanol applied externally, determining the ethanol concentration–response in the presence of intracellular 25 mM 1-pentanol would be equivalent to determining it in the presence of a background concentration of 430 mM ethanol. The predicted effect (E_p) for each ethanol concentration was calculated using the equation:

$$E_p = (E_{(430+x)} - E_{430})/(100 - E_{430}) \quad (3)$$

where x is the ethanol concentration in mM, E_{430} is the percent inhibition by 430 mM ethanol (i.e. 80%), and

$E_{(430+x)}$ is the percent inhibition (from the control ethanol concentration–response curve) produced by (430+x) mM ethanol.

3. Results

One approach to determine whether alcohols act upon an intracellular site on the NMDA receptor-channel would be to test the effect of alcohols in mutant receptor subunits that lack the intracellular regions of the protein. Although it is not possible to remove all residues exposed to the intracellular environment and retain a functional receptor-ion channel, the majority of the intracellular region of the NMDA receptor, which is contained in the C-terminal region following the fourth transmembrane domain, can be removed. Accordingly, C-terminal truncation mutants of the NR1 and NR2B subunits (designated NR1T and NR2BT, respectively) in which most of this region was absent were constructed (Fig. 1). In the NR1T and NR2BT mutant subunits, 32 or 41 residues were exposed to the cytoplasmic environment, respectively (vs. 134 residues in the wild-type NR1 subunit and 676 residues in the wild-type NR2B subunit). NMDA activated a current in each combination of wild-type and mutant subunits that was not grossly different from NMDA-activated current in cells expressing wild-type subunits (Fig. 2). Each combination of wild-type and truncation mutant subunits was also sensitive to ethanol. In four individual cells, the current traces of which are shown in Fig. 2, 100 mM ethanol produced 33–38% inhibition of NMDA-activated current. Concentration–response analysis for ethanol inhibition of NMDA-activated current (Fig. 3) revealed that truncation of the NR1 subunit did not significantly alter the ethanol IC_{50} (149±9.76 mM vs. a control value of 138±8.71 mM; $P>0.05$), whereas truncation of the NR2B subunit produced a slight decrease in the ethanol IC_{50} (107±7.25 mM vs. a control value of 138±8.71 mM; $P<0.01$, ALLFIT analysis, n=6–7). The ethanol sensitivity of NMDA receptors containing the NR1T/NR2BT combination did not differ significantly from that of the NR1/NR2BT combination (92.3±6.61 vs. 107±7.25 mM, respectively; $P>0.05$).

Because C-terminal truncation of the NMDA receptor subunits did not eliminate all residues in the protein exposed to the cytoplasm, experiments were performed in which an alcohol was applied intracellularly by adding it to the patch-pipette solution. If alcohols acted at a site located in the intracellular regions of the protein, applying an alcohol intracellularly would be predicted to decrease or eliminate the effect of an equal or a lower concentration of alcohol applied extracellularly. Nearly maximal inhibitory concentrations for the intracellular alcohol were used in order to have the highest probability of detecting an effect, and 1-pentanol was used

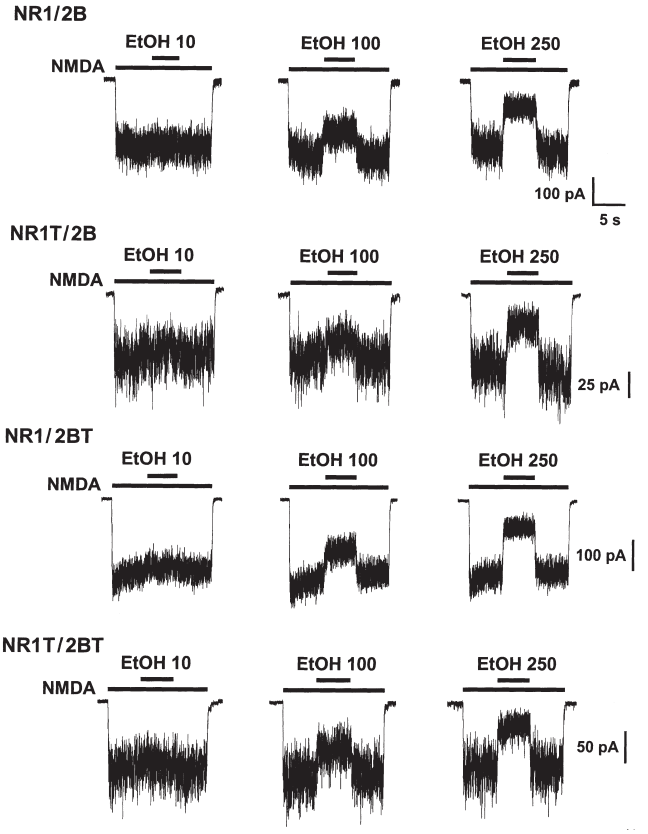

Fig. 2. Current activated by NMDA, and its inhibition by ethanol, in each combination of wild-type and truncation mutant NR1 and NR2B subunits. Traces are currents activated by 25 μM NMDA and 10 μM glycine, and their inhibition by 10, 25, and 100 mM ethanol (EtOH) in NR1/2B, NR1T/2B, NR1/2BT, and NR1T/NR2BT subunit combinations. Time scale for the top row of traces applies to all records.

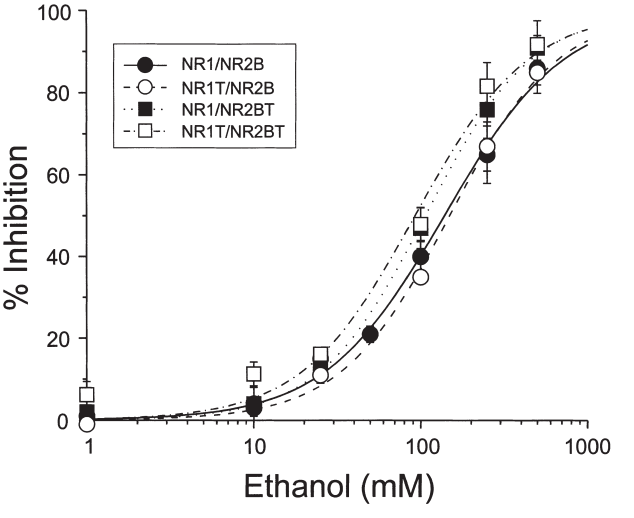

Fig. 3. Concentration–response curves for inhibition of NMDA-activated ion current by ethanol in each combination of wild-type and truncation mutant NR1 and NR2B subunits. Concentrations of NMDA and glycine used were 25 and 10 μM, respectively. Data points are means±S.E.M. of 4–9 cells, and lines shown are least-squares fits to Eq. (2).

instead of ethanol for intracellular application because the high concentrations of ethanol required to produce near-maximal inhibition (250–500 mM; see Fig. 3B) interfered with gigaohm seal formation and were not well tolerated by the cells. Fig. 4A shows traces of current activated by NMDA in the presence of glycine and its inhibition by extracellular 1-pentanol in an individual cell. In this cell, 2.5, 10, and 25 mM 1-pentanol inhibited NMDA-activated current by 17, 48, and 80%, respectively. Concentration–response analysis (Fig. 4B) yielded an IC_{50} value for 1-pentanol of 9.94±1.06 mM, and a slope factor for the concentration–response curve of 1.20±0.137. A concentration of 25 mM 1-pentanol, which produced an average of 80% inhibition of NMDA-activated current, was chosen for intracellular application. When 25 mM 1-pentanol was included in the patch-pipette solution, the inhibitory effect of extracellularly applied 25 mM 1-pentanol on NMDA-activated current was not changed (76±1% inhibition vs. a control value of 84±6% inhibition; $P>0.05$; ANOVA and SNK ranges test, $n=4$; data not shown). The effect of 1-pentanol applied via the patch-pipette on the ethanol concentration–response was also determined. The current traces in Fig. 5A illustrate that ethanol inhibition of NMDA-activated current increased with increasing ethanol concentration, and was comparable whether the patch-pipette contained normal intracellular solution or intracellular solution containing 1-pentanol. Concentration–response analysis of ethanol inhibition of NMDA-activated current (Fig. 5B) revealed that intracellular 1-pentanol did not significantly alter the ethanol IC_{50} (106±9.98 mM vs. a control value of 138±13.2 mM) or the slope factor (1.22±0.133 vs. a control value of 1.22±0.134) of the concentration–response curve ($P>0.05$; ALLFIT analysis, $n=5$–9). Furthermore, the concentration–response curve for ethanol inhibition in the presence of intracellular 25 mM 1-pentanol was considerably to the left of the curve that would be predicted if extracellular ethanol and intracellular 1-pentanol acted at the same site (Fig. 5B, dotted line). Although unlikely, it is possible that 1-pentanol could inhibit NMDA-activated current intracellularly without altering the inhibitory effects of extracellular ethanol or pentanol. As can be seen in Fig. 5C, intracellular 1-pentanol did not alter the normalized amplitude of NMDA-activated current (34.2±9.68 pA/pF vs. a control value of 42.7±15.5 pA/pF; $P>0.05$; Student's t-test, $n=10$).

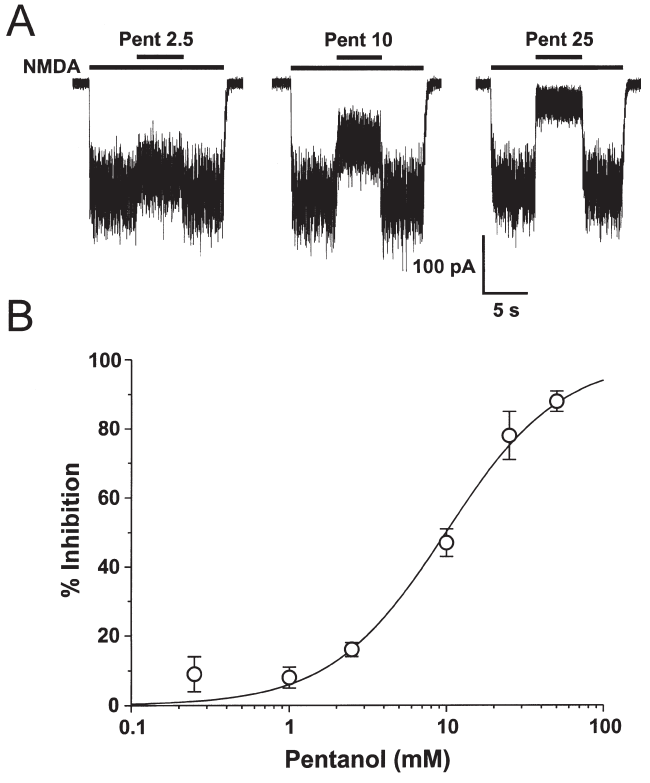

Fig. 4. Inhibition of NMDA-activated ion currents by 1-pentanol. (A) Traces are currents activated by 25 μM NMDA and 10 μM glycine, and their inhibition by 2.5, 10, and 25 mM 1-pentanol (Pent) in an individual cell. (B) Concentration–response for 1-pentanol inhibition of current activated by 25 μM NMDA and 10 μM glycine. Data points are means±S.E.M. of 5–6 cells, and the line shown is the least-squares fit to Eq. (2).

As an additional test to determine whether intracellular 1-pentanol is able to inhibit NMDA-activated current in the absence of extracellular ethanol or 1-pentanol, NMDA was applied at intervals of 30 s immediately after establishing whole-cell recording with 1-pentanol in the patch-pipette solution. This allowed comparison, in the same individual cells, of the amplitude of NMDA-activated current at various times during the process of diffusion of 1-pentanol throughout the cytoplasm. Fig. 6A shows traces of currents activated by 25 μM NMDA and 10 μM glycine at different times after establishing whole-cell recording mode in an individual cell with 25 mM 1-pentanol in the patch-pipette solution. As can be seen, the amplitude of NMDA-activated current did not decrease despite the fact that the intracellular pentanol concentration in this cell increased from 28% to over 99% of the final concentration over the time range shown. Plotting the amplitude of current activated by 25 μM NMDA and 10 μM glycine versus time, expressed as the multiple of the τ_d, for five cells yielded similar results (Fig. 6B). The average amplitude of NMDA-activated current did not change significantly during the time required for diffusion of pentanol throughout the cytoplasm ($P>0.05$; ANOVA, $n=5$).

To ensure that ethanol acts on NMDA receptors in a manner similar to 1-pentanol, and to test more directly whether ethanol could inhibit NMDA receptors via an action at an intracellular site, experiments were performed using cell-attached and inside-out membrane patches. Fig. 7 shows results of an experiment using a cell-attached patch. In this cell, NMDA receptor activity (as assessed by total charge in pC/min) was unaffected

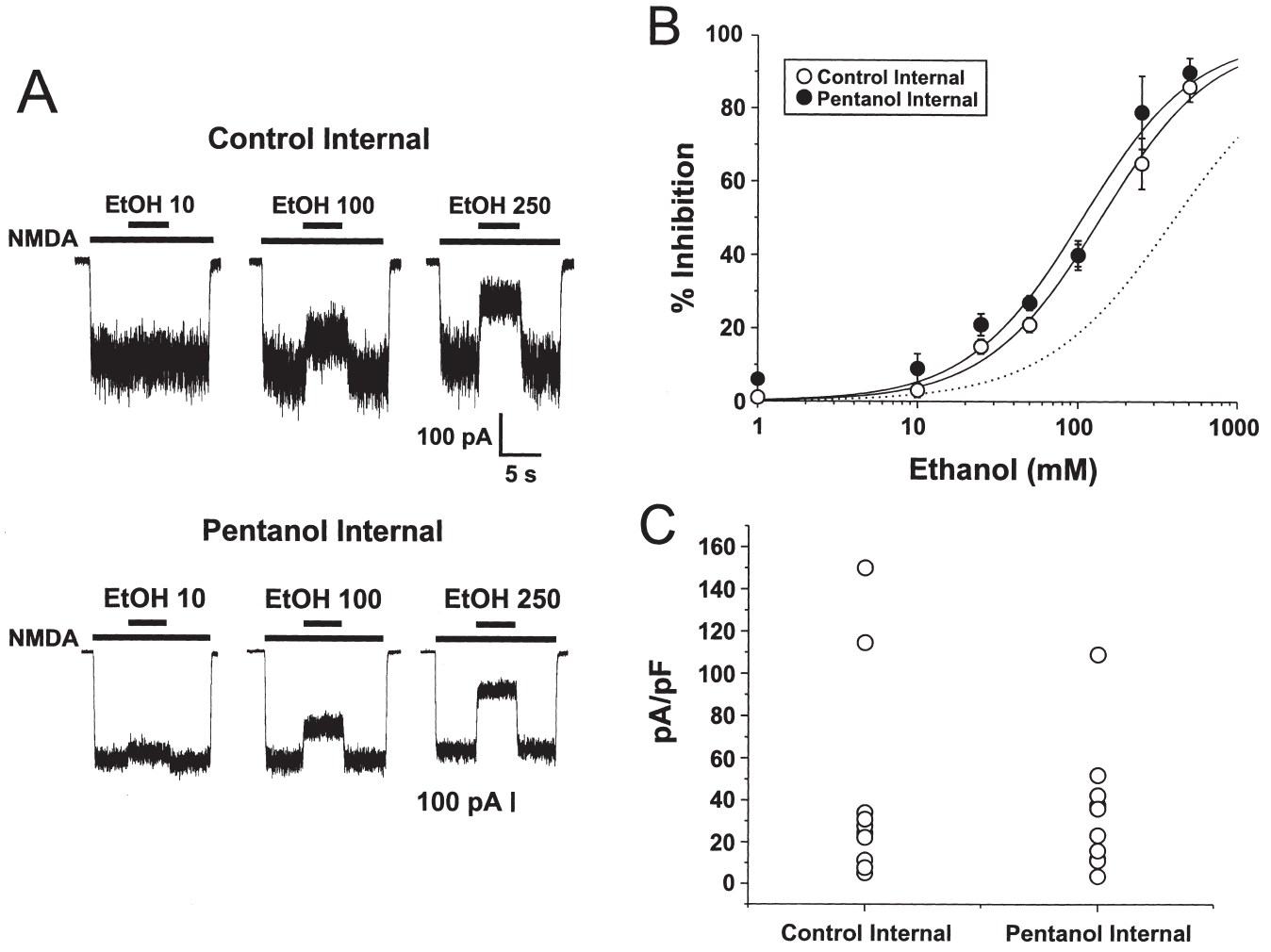

Fig. 5. Effect of intracellular 1-pentanol on inhibition of NMDA-activated current by various concentrations of ethanol. (A) Traces are currents activated by 25 μM NMDA and 10 μM glycine, and their inhibition by 10, 100, and 250 mM ethanol (EtOH) in the absence or the presence of 25 mM 1-pentanol in the patch-pipette solution. Traces shown were obtained from two cells. Time scale for the upper set of traces also applies to the lower set of traces. (B) Concentration–response for ethanol in the absence (open circles) or the presence (closed circles) of 25 mM 1-pentanol in the patch-pipette solution. Data points are means±S.E.M. of 5–9 cells, and the solid lines shown are the least-squares fits to Eq. (2). The dotted line is the predicted ethanol concentration–response curve in the presence of intracellular 1-pentanol, assuming that 1-pentanol could act with equal potency whether applied intra- or extracellularly, determined as described in Section 2. (C) Amplitude of NMDA-activated current, normalized to cell capacitance in pF, in the absence or the presence of 25 mM 1-pentanol in the patch-pipette solution. Each data point is from an individual cell.

by application of ethanol, 100 or 250 mM, to the cell body (i.e. all of the cell membrane with the exception of the patch). The total charge conducted by NMDA receptors in this patch was 105 and 110% of control in the presence of 100 and 250 mM ethanol, respectively; similar results were obtained in five cell-attached patches tested. Results of an experiment in an inside-out patch are shown in Fig. 8. In this patch, NMDA receptor activity (as assessed by total charge in pC/min) was unaffected by application of ethanol, 100 or 250 mM, directly to the cytoplasmic face of the patch. The total charge conducted by NMDA receptors in this patch was 103 and 104% of control in the presence of 100 and 250 mM ethanol, respectively; similar results were obtained in four cell-attached patches tested.

4. Discussion

Since the initial observations of alcohol inhibition of NMDA receptor-channels (Göthert and Fink, 1989; Hoffman et al., 1989; Lovinger et al., 1989), the site of action of alcohols on NMDA receptor-channels has remained elusive. Studies conducted in an attempt to observe interactions of ethanol with known sites on the NMDA receptor-channel, such as the agonist binding site (Hoffman et al., 1989; Gonzales and Woodward, 1990; Rabe and Tabakoff, 1990; Peoples et al., 1997), and the sites for various modulators of receptor-channel function (Rabe and Tabakoff, 1990; Woodward and Gonzales, 1990; Chu et al., 1995; Bhave et al., 1996; Peoples et al., 1997), have not yet identified a specific

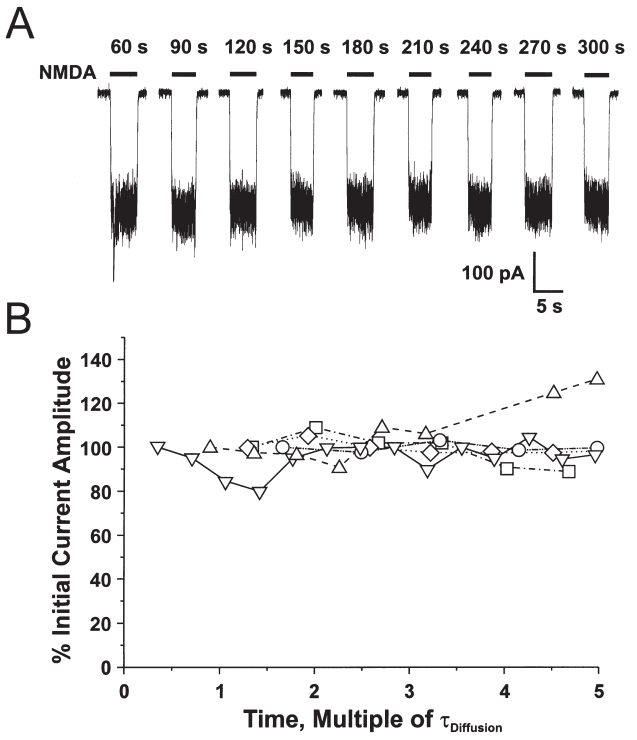

Fig. 6. Effect of diffusion of 1-pentanol throughout the intracellular compartment on the amplitude of NMDA-activated current. (A) Traces are currents activated by 25 μM NMDA and 10 μM glycine at different times (in seconds) after establishing whole-cell recording mode with 25 mM 1-pentanol in the patch-pipette solution. Traces shown were obtained from an individual cell. The initial and final traces shown correspond to 1.3 and 6.4 times the τ_d for this cell, respectively. (B) Amplitude of current activated by 25 μM NMDA and 10 μM glycine, expressed as the percent of the initial current amplitude, versus time, expressed as the multiple of the τ_d. Values of τ_d were calculated for each cell using Eq. (1). Data points represented by a given symbol are values from an individual cell, and lines shown are point-to-point fits.

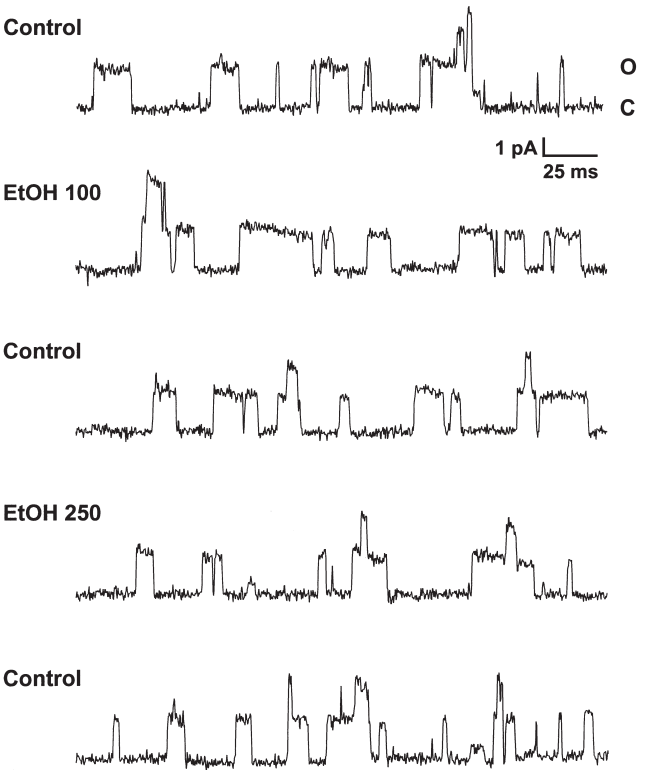

Fig. 7. Effect of ethanol on NMDA receptor activity in cell-attached patches. Traces are single-channel currents activated by 100 μM NMDA and 10 μM glycine in a cell-attached patch from a single cell. Traces are representative segments of 30 s records, and are sequential from top to bottom. Each cell was continuously bathed in extracellular solution, either in the absence or the presence of ethanol (EtOH) at 100 or 250 mM. Similar results were obtained in five patches tested.

site of alcohol action. The possibility that alcohol inhibits NMDA receptors via an open-channel blocking mechanism has been excluded based on a study using single-channel recording (Wright et al., 1996), in which ethanol was observed to decrease mean open time and frequency of opening of the ion channel without producing flickering block or affecting channel amplitude. Studies addressing a possible interaction of alcohols with the glycine coagonist site have in some cases reported such an interaction (Hoffman et al., 1989; Rabe and Tabakoff, 1990; Woodward and Gonzales, 1990; Dildy-Mayfield and Leslie, 1991; Buller et al., 1995; Popp et al., 1999), while in other studies no such interaction has been observed (Gonzales and Woodward, 1990; Peoples and Weight, 1992; Woodward, 1994; Chu et al., 1995; Mirshahi and Woodward, 1995; Cebers et al., 1996; Peoples et al., 1997). The reasons for these discrepant results are not yet entirely clear, but may in part involve differences in NMDA receptor subunit composition (Buller et al., 1995), as well as differences in intracellular modulators that depend upon cell type and experimental protocol. For example, a recent study performed in cultured rat cerebellar granule neurons reported that glycine reversal of inhibition by 100 mM ethanol of NMDA-activated steady-state current was not observed in recordings using the whole-cell patch-clamp recording mode, but was pronounced when the perforated-patch recording mode was used (Popp et al., 1999). This study also reported that ethanol inhibition of peak NMDA-activated current was enhanced by pre-exposure to ethanol in these neurons. Both of these findings would seem to suggest that one or more intracellular factors, such as a protein kinase (Snell et al., 1994), contribute significantly to ethanol inhibition, and its reversal by glycine, in this cell type. Thus these intracellular factors, rather than the glycine coagonist site, may constitute additional target sites for ethanol action not physically located on the NMDA receptor-channel complex that account for the glycine reversal phenomenon.

The observations of the present study that C-terminal truncation did not eliminate or reduce ethanol sensitivity of the NR1 subunit, and slightly increased ethanol sensitivity of the NR2B subunit, would argue against the intracellular C-terminus as the location of the site of action of alcohol, and may instead indicate that the C-

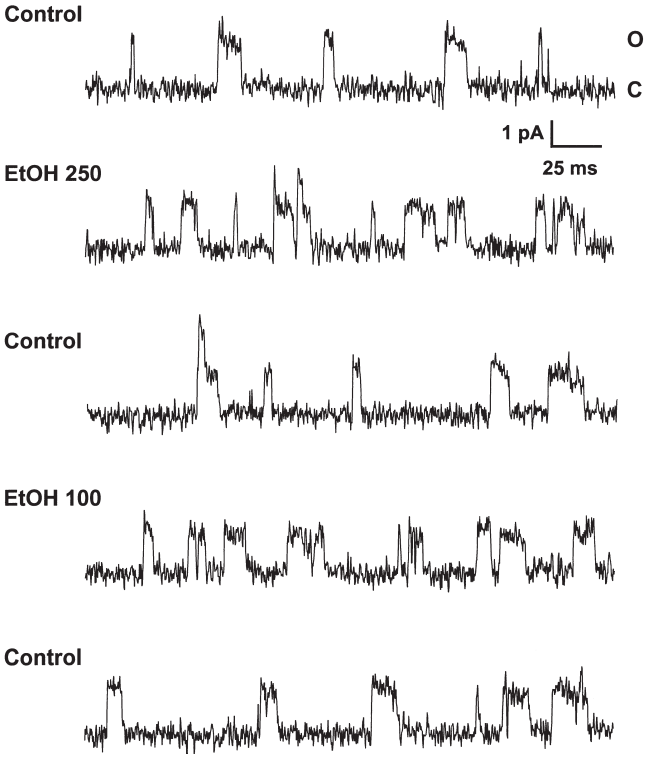

Fig. 8. Effect of ethanol on NMDA receptor activity in inside-out patches. Traces are single-channel currents activated by 100 μM NMDA and 10 μM glycine in an inside-out patch from a single cell. Traces are representative segments of 30 s records, and are sequential from top to bottom. The cytoplasmic face of each patch was continuously bathed in extracellular solution, either in the absence or the presence of ethanol (EtOH) at 100 or 250 mM. Similar results were obtained in four patches tested.

terminal domain of the NR2B subunit exerts a slight negative regulatory effect on ethanol sensitivity under normal conditions. The C-terminal domain of NR1 contains binding sites for a number of intracellular proteins, including calmodulin (Zhang et al., 1998; Krupp et al., 1999) and the anchoring/scaffold proteins α-actinin-2 (Krupp et al., 1999) and yotiao (Lin et al., 1998), as well as phosphorylation sites for protein kinases A and C (Tingley et al., 1997). Similarly, the NR2B C-terminal domain possesses binding sites for the anchoring proteins PSD-95 (Kornau et al., 1995; Niethammer et al., 1996) and SAP-102 (Lau et al., 1996), sites for phosphorylation by calcium/calmodulin-dependent protein kinase II (Zhang et al., 1998) and protein tyrosine kinases (Moon et al., 1994), and a consensus phosphorylation site for protein kinase A. All of the above sites were absent from the NR1 and NR2B truncation mutant subunits in the present study. Thus the results of the present study are not consistent with the view that alcohol inhibition of NMDA receptors is mediated primarily by an interaction of alcohol with intracellular regulatory proteins or second messenger systems, which then interact with the NMDA receptor C-terminal domain.

Experiments in which alcohols were applied selectively to either the extracellular or intracellular side of the membrane were also consistent with an action of alcohols on the extracellular region of the NMDA receptor protein. Because the alcohols would have access to the cytoplasmic, but not the extracellular, regions of the receptor under the conditions used, the findings of the present study are not consistent with an action of alcohols on the cytoplasmic regions of the receptor. In addition, under the conditions used in these experiments the alcohols would have access to residues in the transmembrane regions of the receptor protein that are exposed to the surrounding membrane lipids. If the site of alcohol action is located in a transmembrane domain, as appears to be the case for sites of alcohol action on γ-aminobutyric acid and glycine receptors (Mihic et al., 1997; Wick et al., 1998), the present results would suggest that this site is accessible only from the extracellular environment, and is not accessible from within the membrane. The results of the present study are thus most consistent with an action of alcohols on a site located on the NMDA receptor in a region that is exposed to, or only accessible from, the extracellular environment.

The results of the present study do not identify a specific site of alcohol action on the NMDA receptor, but they do provide important information about the existence and location of such a site. Inhibition of NMDA receptor-channels in mouse hippocampal neurons by *n*-alcohols exhibits a cutoff effect (Peoples and Weight, 1995), but this lack of interaction of higher alcohols with the NMDA receptor appears to be attributable primarily to an inability to achieve adequate aqueous concentrations, rather than to an inability to bind to the alcohol site due to their physical dimensions (Peoples, 1999). The results of the present study thus provide the clearest evidence to date for a direct interaction of alcohols with the NMDA receptor protein. Because alcohols readily partition into and cross the cell membrane regardless of whether they are applied to the extracellular or intracellular side of the membrane, the observations in the present study that alcohols inhibit NMDA receptor function only when applied to the extracellular side of the membrane are exceedingly difficult, if not impossible, to explain in terms of an action of alcohols on the cell membrane lipids or on a separate cytoplasmic protein that interacts with the NMDA receptor.

It is important to note that the findings of the present study refer to the site through which alcohol inhibits the NMDA receptor, rather than to sites on the receptor-channel that may regulate its alcohol sensitivity. Although alcohols undoubtedly interact with multiple intracellular second messenger systems (Diamond and Gordon, 1997), and these interactions may modulate the ethanol sensitivity of the NMDA receptor (Snell et al., 1994; Miyakawa et al., 1997), these interactions are neither necessary nor sufficient to explain alcohol inhibition of NMDA receptor function. The observations of

the present study thus do not conflict with results of recent studies demonstrating that the intracellular, C-terminal domain of the NMDA receptor-channel is involved in the regulation of alcohol sensitivity (Mirshahi et al., 1998; Anders et al., 1999). In these previous studies, ethanol sensitivity of NMDA receptors composed of NR1/NR2A subunits was reduced slightly by Fyn tyrosine kinase phosphorylation of the NR2A subunit (Anders et al., 1999), and an increase in ethanol sensitivity produced by high extracellular calcium was abolished by truncation of the C-terminus of the NR1 subunit. The finding in the present study that truncation of the intracellular C-terminal domain of the NR2B subunit slightly increased ethanol sensitivity would appear to be consistent with a role of the C-terminus of NMDA receptor subunits in regulating ethanol sensitivity, although this regulation appears to differ among subunits and to depend upon experimental conditions. It is possible that alterations in the receptor-channel protein produced at sites distant from the alcohol binding site could influence the presumed conformational change produced in the receptor-channel by alcohol binding, resulting in an altered effect of alcohol. Further studies will be needed to precisely identify the specific alcohol binding site or sites on the NMDA receptor protein, as well as the molecular mechanisms through which alcohols modulate its activity.

Acknowledgements

We thank Chaoying Li, Forrest Weight, and Mark Mayer for helpful discussions and comments on the manuscript, and Amir Ghazanfari for technical assistance. This research was supported by the intramural program of the National Institute on Alcohol Abuse and Alcoholism, NIH.

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