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Inhibition of *N*-Methyl-D-aspartate Receptors by Straight-Chain Diols: Implications for the Mechanism of the Alcohol Cutoff Effect

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ABSTRACT

n-Alkanol inhibition of *N*-methyl-D-aspartate (NMDA) receptors exhibits a "cutoff" effect: alcohols with up to eight to nine carbon atoms inhibit the receptor, whereas larger alcohols do not. This phenomenon was originally proposed to result from size exclusion; i.e., alcohols above the cutoff are too large to bind to an amphiphilic site on the receptor. In the present study, 1,Ω-diols with 3 to 14 carbon atoms inhibited NMDA-activated current in Chinese hamster ovary and human embryonic kidney 293 cells transiently expressing NR1 and NR2B NMDA receptor subunits. Results of fluctuation analysis experiments were consistent with a similar mechanism of inhibition of NMDA-activated current by alcohols and diols. The average change in apparent energy of binding of the diols caused by addition of a methylene group was 2.1 kJ/mol, which is consistent with an important role of hydrophobic interactions. Because 1,Ω-diols with 9 to 14 carbons inhibited NMDA-activated current, despite having molecular volumes exceeding that at the cutoff point for 1-alkanols, a size exclusion mechanism seems inadequate to explain the cutoff effect. A disparity in hydrophobicity values at the cutoff for alcohols and diols, however, revealed that hydrophobicity could also not entirely explain the cutoff phenomenon. From these results, it seems that the cutoff effect on NMDA receptors results primarily from the inability of long-chain alcohols to achieve adequate concentrations at their site of action due to low aqueous solubility, although other factors may also contribute to the effect.

N-Methyl-D-aspartate (NMDA) receptor-ion channels are believed to be important targets of alcohol action in the central nervous system. Previous studies from this and other laboratories have demonstrated that *n*-alcohol inhibition of NMDA receptor-ion channels exhibits a "cutoff" effect: as a series of straight-chain alcohols is ascended, inhibitory potency of the alcohols increases up to a chain length of eight to nine carbon atoms then declines beyond the cutoff point and eventually disappears (Peoples and Weight, 1995; Dildy-Mayfield et al., 1996). Cutoff phenomena for alcohol effects on other receptors (Moody et al., 1991; Murrell et al., 1991; Li et al., 1994; Mascia et al., 1996; Mitchell et al., 1996), soluble proteins (Franks and Lieb, 1985), and lipid membranes (Lee, 1976; Lyon et al., 1981; Chiou et al., 1990) have also been observed, although these generally occur at higher chain lengths (typically at 12–13 carbon atoms), and several explanations for these effects have been suggested (Peoples et al., 1996). The cutoff for NMDA receptor inhibition was originally proposed to result from size exclusion (Peoples and Weight, 1995); i.e., alcohols act by binding to an amphiphilic region on the receptor-ion channel protein, and alcohols above the cutoff are unable to bind because their size exceeds the dimensions of the site. A limitation inherent in using a series of *n*-alcohols of varying lengths is that both molecular volume and hydrophobicity of alcohols increase concurrently with increasing carbon chain length, making it impossible to determine the relative contributions of these factors to the cutoff effect. In the present study, 1,Ω- and 1,2-*n*-diols were used as analogs of 1-*n*-alcohols to evaluate the roles of molecular volume and hydrophobicity in the cutoff for NMDA receptor inhibition. The additional hydroxyl group of the diols results in a greatly reduced hydrophobicity but slightly increased molecular volume relative to the alcohols.

Experimental Procedures

Materials. Ethanol (95%, prepared from grain) was obtained from Pharmco (Brookfield, CT); all other alcohols and diols were obtained from Aldrich Chemical (Milwaukee, WI). Ketamine was obtained from Sigma/RBI (Natick, MA), and all other drugs were obtained from Sigma Chemical (St. Louis, MO). Representative structures of a 1-*n*-alcohol, a 1,Ω-*n*-diol, and a 1,2-*n*-diol are shown in Fig. 1.

Cell Culture. Chinese hamster ovary (CHO) K1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in a medium consisting of Ham's F-12K nutrient

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CHO, Chinese hamster ovary; HEK, human embryonic kidney; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ANOVA, analysis of variance.

mixture containing 2 mM *l*-glutamine and 1.5 g/l 90% sodium bicarbonate, and 10% fetal bovine serum. Human embryonic kidney (HEK) 293 cells obtained from the American Type Culture Collection were cultured in medium consisting of minimum essential medium containing 2 mM *l*-glutamine, Earle's balanced salt solution, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% heat-inactivated horse serum. CHO cells were used in all experiments, and both CHO and HEK 293 cells were used in fluctuation analysis experiments.

Transient Transfection. NMDA receptor cDNA clones for the rat NR1–1a and NR2B subunits were gifts from Drs. D. R. Lynch (University of Pennsylvania, Philadelphia, PA) and D. M. Lovinger (Vanderbilt University, Nashville, TN). Cells were seeded in 35-mm dishes and allowed to grow to 70 to 95% confluence; they were then transfected with cDNA for the NR1 and NR2B subunits and green fluorescent protein (pGreen Lantern; Invitrogen, Carlsbad, CA) in a 2:2:1 ratio, respectively, by using LipofectAMINE PLUS or LipofectAMINE 2000 (Invitrogen). The culture medium during and after the transfection step contained 100 μM ketamine and 200 μM *dl*-2-amino-5-phosphonovaleric acid to minimize cell death caused by excitotoxicity. CHO cells were mechanically dissociated 18 to 48 h after transfection, and were replated at low density (~10–30% confluence) on polyornithine-coated 35-mm dishes at least 1 h before recording; HEK 293 cells were not dissociated and replated before recording.

Electrophysiological Recording. Patch-clamp recording of whole-cell currents was performed at room temperature by using an Axopatch 200 or 200B (Axon Instruments, Foster City, CA) amplifier. Gigaohm seals were formed using electrodes with tip resistances of 2 to 5 MΩ, and series resistances of 4 to 15 MΩ were compensated by 80%. Cells were voltage-clamped at −50 mV, unless noted otherwise. Data were filtered (0.2–2 kHz; low-pass, eight-pole Bessel) and acquired at 1 to 5 kHz on a computer by using a DigiData interface and pClamp software (Axon Instruments). In fluctuation analysis experiments, data were recorded on videotape by using a VR-10B digital data recorder (Instrutech, Great Neck, NY) connected to a videocassette recorder (Sony SLV-440). Data were later replayed through a low-pass, eight-pole Butterworth filter (1-kHz corner frequency) and 25 to 60 traces of length 600 ms were acquired at 5 kHz on a computer. Traces were averaged and analyzed for each treatment condition in each cell.

Solutions. Cells were superfused at 1 to 2 ml/min in an extracellular medium containing 150 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose; pH was adjusted to 7.4 with NaOH and osmolality to 340 mmol/kg with sucrose. Low Ca^{2+} was used to minimize NMDA receptor inactivation (Zilberter et al., 1991). The patch-pipette solution contained 140 mM CsCl, 2 mM Mg_4ATP, 10 mM BAPTA, and 10 mM HEPES; pH was adjusted to 7.4 with CsOH and osmolality to 310 mmol/kg with sucrose. Solutions of agonists, alcohols, and diols were prepared fresh daily in extracellular solution. Solutions of long-chain alcohols (>9 carbon atoms) or diols (>10 carbon atoms) were prepared from stock solutions in 95% ethanol, resulting in final ethanol concentrations of ≤1.6 mM (with the exception of 0.1 mM 1,12-dodecanediol, in which the final ethanol concentration was 6.5 mM); the same amount of ethanol was added to control solutions. Solutions of agonists and drugs were applied to cells with a rapid solution exchange apparatus (Li et al., 1998). Solutions containing excitatory amino acids were applied at intervals of at least 90 s, unless noted otherwise.

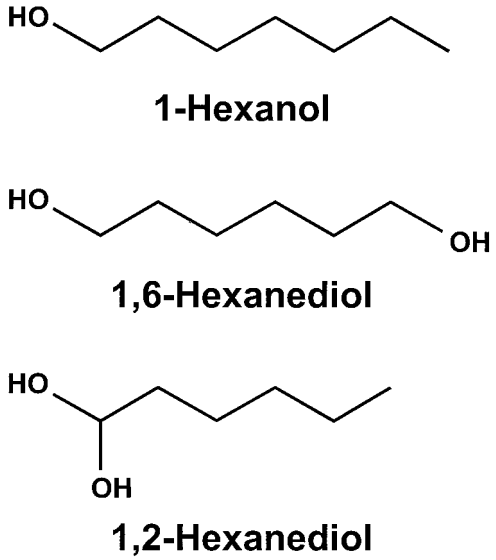

Fig. 1. Representative alcohol and diol structures. 1-Hexanol is shown as an example of a straight-chain primary alcohol, and 1,6-hexanediol and 1,2-hexanediol are shown as examples of 1,Ω- and 1,2-diols, respectively.

Calculation of Physical Properties. Log octanol/water partition coefficient (log P) values were estimated using the program log P calculator (Advanced Chemistry Development, Inc., Toronto, ON, Canada). Molecular (van der Waals) volumes were calculated using Spartan Pro (Wavefunction, Irvine, CA) after structural optimization by using the AM1 semiempirical parameters. Saturating concentrations of alcohols and alkanes in water (C_{sat}) were those reported by Bell (1973). Because plots of log C_{sat} versus log P for *n*-alcohols and *n*-alkanes were linear and differed only minimally (respective slopes −1.09 versus −1.26; respective *y*-intercepts 0.935 versus 1.08), values of C_{sat} for diols were estimated using the equation:

$$\log C_{sat} = -1.18 \log P + 1.01 \quad (1)$$

which was derived from a least-squares fit to the plot of log C_{sat} versus log P for *n*-alkanes and *n*-alcohols.

Calculation of Apparent Binding Energies. Change in apparent energy of binding (ΔΔG) upon addition of a methylene group to a diol was calculated using the equation:

$$\Delta\Delta G = -RT \ln[(IC_{50})_n/(IC_{50})_{n+1}] \quad (2)$$

where n is the number of carbon atoms in the diol, R is the gas constant, T is the temperature in °K, and IC_{50} is the half-maximal inhibitory concentration (Franks and Lieb, 1985).

Curve Fitting and Statistical Analysis. Percentage of inhibition by alcohols and diols was calculated using the average of the control NMDA-activated currents before and after the test response, with the exception of the highest concentrations of octanol and nonanol, in which cases only the pre-exposure control values were used because of delayed recovery from inhibition. 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:

$$y = E_{max}/1 + (IC_{50}/x)^n \quad (3)$$

where x and y are concentration and response (e.g., percentage of inhibition), respectively, and E_{max} is the maximal response. IC_{50} values obtained from the curve fits for dodecanediol and tetradecanediol should be considered to be estimates because the highest concentrations of these diols that could be tested produced less than 50% inhibition; in addition, in the case of tetradecanediol, it was necessary to constrain the slope factor to the average of the other curves to obtain an adequate fit. In noise analysis experiments, fast Fourier transformations of the data were performed using the program Clampfit 8.0 (Axon Instruments), background spectra were subtracted from spectra for NMDA or NMDA and hexanediol, and the data were fitted with a Lorentzian function of the form:

$$S_f = S_o[1/1 + (f/f_c)^2] \quad (4)$$

where S_f is the spectral density at frequency f (in Hz), S_o is the zero-frequency asymptote, and f_c is the corner frequency. Time constants (τ) were obtained from the relationship $\tau = 1/2\pi f_c$. In variance analysis experiments, mean current amplitude and variance of the data were obtained using the program Clampfit 8.0. Statistical evaluation of differences among means was determined by ANOVA or Student's t tests by using the program InStat (GraphPad Software, San Diego, CA). All values are reported as mean ± S.E.M.

Results

Figure 2 shows inhibition of NR1/NR2B NMDA receptor-mediated currents by a series of straight-chain primary alcohols. As can be seen, alcohols up to 1-nonanol inhibited NMDA-activated current, whereas 1-decanol, even at the highest concentration that could be tested, produced no observable inhibition (Fig. 2a). As the series of alcohols was ascended up to 1-nonanol, the concentration-response curves were shifted progressively to the left in a parallel manner (Fig. 2b).

1,Ω-n-Diols from 1,3-propanediol to 1,14-tetradecanediol inhibited NMDA-activated current in CHO cells expressing NR1/NR2B NMDA receptor subunits (Fig. 3a). Inhibition by diols was rapid in onset and offset and was qualitatively indistinguishable from the inhibition produced by various alcohols. As the carbon chain length of the diols increased, decreasing concentrations of the diols produced roughly similar magnitudes of inhibition. In contrast to diols of shorter carbon chain length, however, 1,12-dodecanediol and 1,14-tetradecanediol produced little or no inhibition at the highest concentrations that could be tested. Inhibition of NMDA receptors by diols was concentration-dependent (Fig. 3b). In-

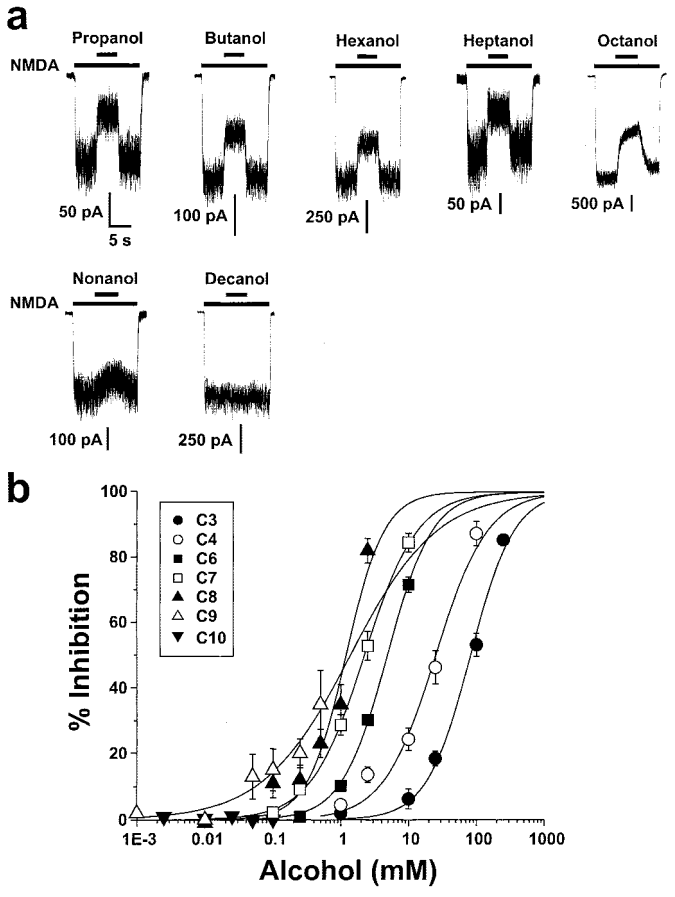

Fig. 2. Effect of 1-alcohols on NMDA receptors. a, records of current activated by 25 μM NMDA and 10 μM glycine and its modulation by various 1-alcohols. Concentrations of alcohols were 100 mM 1-propanol, 25 mM 1-butanol, 2.5 mM 1-hexanol, 2.5 mM 1-heptanol, 1 mM 1-octanol, 0.5 mM 1-nonanol, and 0.1 mM 1-decanol. b, concentration-response curves for inhibition of current activated by 25 μM NMDA and 10 μM glycine by 1-alkanols from 1-propanol to 1-decanol. Results are means of n = 4 to 8 CHO cells. Curves shown are fits to eq. 3 under *Experimental Procedures*. Respective IC_{50} and slope factor values were 1-propanol, 82.3 ± 6.07 mM and 1.36 ± 0.124; 1-butanol, 25.3 ± 3.42 mM and 1.14 ± 0.175; 1-hexanol, 4.88 ± 0.156 mM and 1.31 ± 0.0491; 1-heptanol, 2.24 ± 0.0508 mM and 1.11 ± 0.0283; 1-octanol, 1.2 ± 0.16 mM and 1.54 ± 0.306; and 1-nonanol, 1.39 ± 0.479 mM and 0.68 ± 0.126. Respective IC_{50} and slope factor values for ethanol of 138 ± 7.38 mM and 1.22 ± 0.0749 and for 1-pentanol of 9.94 ± 1.06 mM and 1.2 ± 0.137 were determined in a previous study (Peoples and Stewart, 2000).

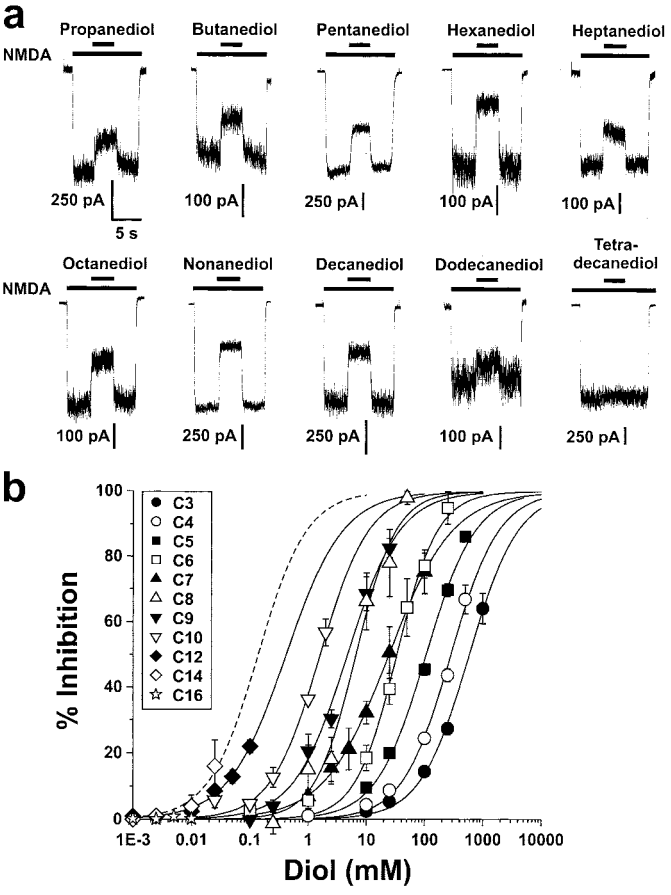

Fig. 3. Effect of 1,Ω-diols on NMDA receptors. a, records of current activated by 25 μM NMDA and 10 μM glycine and its modulation by various diols. Concentrations of diols were 500 mM 1,3-propanediol, 250 mM 1,4-butanediol, 250 mM 1,5-pentanediol, 100 mM 1,6-hexanediol, 25 mM 1,7-heptanediol, 10 mM 1,8-octanediol, 1 mM 1,9-nonanediol, 2 mM 1,10-decanediol, 0.1 mM 1,12-dodecanediol, and 0.025 mM 1,14-tetradecanediol. b, concentration-response curves for inhibition of current activated by 25 μM NMDA and 10 μM glycine by various diols from 1,3-propanediol to 1,16-hexadecanediol. Results are means of n = 4 to 13 CHO cells. Curves shown are fits to eq. 3 under *Experimental Procedures*. Respective IC_{50} and slope factor values were 1,3-propanediol, 592 ± 30.7 mM and 1.04 ± 0.0553; 1,4-butanediol, 286 ± 16.1 mM and 1.06 ± 0.0787; 1,5-pentanediol, 107 ± 7.56 mM and 1.03 ± 0.0715; 1,6-hexanediol, 33.4 ± 2.03 mM and 1.24 ± 0.0979; 1,7-heptanediol, 24.6 ± 0.717 mM and 0.794 ± 0.0185; 1,8-octanediol, 6.60 ± 0.962 mM and 1.25 ± 0.184; 1,9-nonanediol, 4.96 ± 0.381 mM and 0.999 ± 0.0690; 1,10-decanediol, 1.61 ± 0.128 mM and 1.04 ± 0.105; 1,12-dodecanediol, 0.419 ± 0.0684 mM and 0.887 ± 0.0761; and 1,14-tetradecanediol, 138 ± 16.3 μM. The curve for tetradecanediol is shown as a dashed line to indicate that the slope factor for this fit was constrained to the average of the other curves.

creasing the diol carbon chain length progressively shifted the concentration-response curves to the left in a parallel manner, until an apparent maximum was reached at 12 to 14 carbon atoms. 1,16-Hexadecanediol did not inhibit NMDA-activated current at any concentration tested. As the series of diols was ascended above octanol, the highest concentrations of the diols that were soluble in the extracellular solution produced progressively lower values of maximal inhibition. Thus, a nearly maximal concentration of 1,10-decanediol produced an average of 57% inhibition, whereas nearly maximal concentrations of 1,12-dodecanediol and 1,14-tetradecanediol produced only 22 and 10% inhibition, respectively.

As a means of assessing whether diols inhibit NMDA receptors in a manner similar to alcohols, the effects of a typical diol, 1,6-hexanediol, on single-channel characteristics were determined using fluctuation analysis. Figure 4 illustrates that hexanediol, at a concentration of 35 mM, inhibited NMDA-activated current in a manner that seemed to involve a reduction in the amplitude of the current noise associated with channel opening (Fig. 4a). Fitting of Lorenztian functions to power density spectra revealed that 1,6-hexanediol decreased the time constant of the noise underlying the NMDA-activated current (an approximation of the mean open time of the channel; Fig. 4b). On average, the time constants for the noise associated with NMDA-activated current were 4.8 ± 0.24 and 3.8 ± 0.25 ms in the absence and the presence of 35 mM 1,6-hexanediol, respectively; these values differed significantly (t test; $p < 0.05$; $n = 5$ cells).

Variance analysis of current activated by NMDA at different holding potentials additionally revealed that hexanediol did not alter the slope of the line fitted to a plot of unitary current amplitude versus holding potential, indicating that the unitary conductance was unchanged (Fig. 4c). On average, the unitary conductance activated by NMDA was 50 ± 4.7 and 47 ± 4.4 pS in the absence and the presence of 35 mM 1,6-hexanediol, respectively; these results did not differ significantly (t test; $p > 0.05$; $n = 6$ cells). The results of both noise and variance analysis experiments were consistent with the results obtained in a previous study for inhibition of NMDA receptors by ethanol, in which approximately 50% of the inhibitory effect of ethanol was attributable to a decrease in the mean open time of the channel, and in which ethanol did not alter the unitary conductance of the channel (Wright et al., 1996).

To determine whether hydrophobic interactions could account for the increase in NMDA receptor inhibitory potency that was observed as the series of diols was ascended, the apparent change in energy of binding ($\Delta\Delta G$) of the diols to their site of action due to the addition of a methylene group was calculated (Table 1). The average value of $\Delta\Delta G$ for diols with three to nine carbon atoms was -2.09 ± 0.387 kJ/mol, which did not differ significantly from the average value of -1.96 ± 0.240 for addition of a methylene group to a series of n-alcohols (ANOVA; $p > 0.05$), and agrees well with the value of 2.18 kJ/mol predicted for transfer of a methylene group to a hydrophobic binding site on a protein (Nozaki and Tanford, 1971).

To test whether the position of the hydroxyl groups on the hydrocarbon chain could alter diol inhibition of NMDA receptors, the effects of 1,2-diols with 6, 8, and 10 carbon atoms were compared with the corresponding 1,Ω-diols. Figure 5 illustrates that each of the 1,2-diols tested inhibited NMDA-activated current. At the same concentrations, 1,2-hexanediol and 1,2-octanediol produced a slightly greater degree of inhibition compared with the corresponding 1,Ω-diols, whereas 1,2-decanediol and 1,10-decanediol produced a similar degree of inhibition (Fig. 5a). Concentration-response curves for inhibition of NMDA-activated current by the 1,2-diols were approximately parallel to those obtained for the 1,Ω-diols (Fig. 5b), because the slope factors of the fitted curves did not differ significantly (ANOVA; $p > 0.05$); however, the curves for 1,2-hexanediol and 1,2-octanediol were shifted to the left of those for the corresponding 1,Ω-diols. Comparison of the IC_{50} values for the 1,2-diols obtained from concentration-response analysis (Fig. 6a) with those obtained

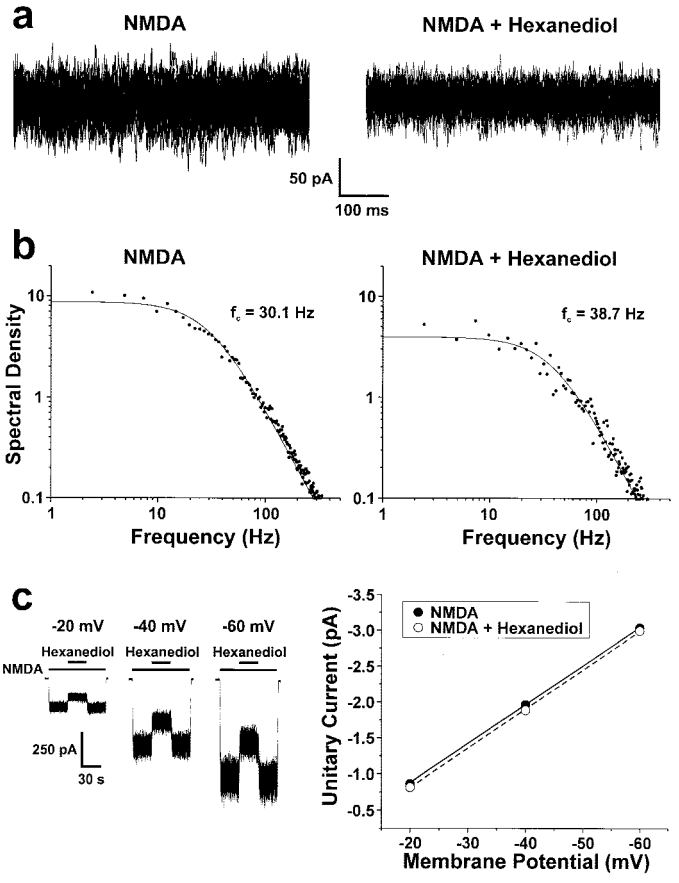

Fig. 4. Noise and variance analysis of 1,6-hexanediol inhibition of NMDA receptors. a, records are 30 overlaid sweeps 600 ms in length of current activated by 25 μM NMDA and 10 μM glycine and its inhibition by 35 mM 1,6-hexanediol in an HEK 293 cell. Note the apparent decrease in the amplitude of the noise produced by 1,6-hexanediol. b, power density spectra of data shown in a of current activated by NMDA and glycine in the absence and the presence of 1,6-hexanediol. Data sweeps were averaged before fast Fourier transformation. Curves shown are the best fits of the data to the Lorentzian function (eq. 4) under *Experimental Procedures*. The corner frequencies of 30.1 and 38.7 Hz obtained for NMDA-activated current in the respective absence and the presence of 1,6-hexanediol correspond to time constants of 5.3 and 4.1 ms. Similar results were obtained in three other HEK 293 cells. c, records (left) are of current activated by 25 μM NMDA and 10 μM glycine and its inhibition by 35 mM 1,6-hexanediol in an HEK 293 cell at holding potentials of -20, -40, and -60 mV. Graph (right) plots the unitary NMDA-activated current versus holding potential in the absence and the presence of 1,6-hexanediol. Each point represents the averaged values from 30 to 60 data sweeps. The solid and dashed lines shown are least-squares fits to the data for NMDA in the absence and the presence of 1,6-hexanediol, respectively. Linear fits yielded slope values of 54 and 55 pS for NMDA and NMDA in the presence of 1,6-hexanediol, respectively.

for the corresponding 1,Ω-diols revealed that 1,2-decanediol did not differ significantly in NMDA receptor inhibitory potency from 1,10-decanediol (ANOVA; $p > 0.05$), but 1,2-hexanediol and 1,2-octanediol were 1.95- and 1.66-fold more potent, respectively, than their corresponding 1,Ω-diols (ANOVA; $p < 0.01$). These differences in potency could be attributed primarily to differences in hydrophobicity, however, as is apparent from a plot of NMDA receptor inhibitory potency versus the log of the octanol/water partition coefficient (log P; Fig. 6b).

To assess the relative contributions of molecular volume and hydrophobicity to the cutoff effect, the potency of alcohols and diols for inhibition of NMDA-activated current was plotted as a function of both van der Waals volume and log P (Fig. 7). As can be seen, the plots of NMDA receptor potency versus molecular volume for both alcohols and diols were linear below the cutoff points and were parallel (Fig. 7a), although the *y*-intercepts of the plots differed considerably (−0.151 versus −1.31 for alcohols and diols, respectively). In contrast to the results obtained for alcohols, however, the NMDA receptor inhibitory potency of diols continued to increase well beyond the molecular volume of 1-nonanol (213.8 Å^3). Plotting NMDA receptor potency as a function of hydrophobicity also yielded parallel lines below the cutoff points for both alcohols and diols (Fig. 7b). The position of the plots for alcohols and diols differed depending upon whether NMDA receptor potency was plotted against molecular volume or hydrophobicity, so that alcohols were more potent than diols at a given molecular volume, whereas the reverse was true at a given log P value. In addition, the log P value of the largest diol that exhibited NMDA receptor inhibitory activity exceeded that of the highest alcohol retaining inhibitory activity. Plotting the IC_{50} values and the saturating aqueous concentrations of the alcohols and diols against their log P values revealed that the highest member of each series of compounds that retained NMDA receptor inhibitory activity had an IC_{50} value approximate to the saturating aqueous concentration (Fig. 8).

Discussion

A large body of evidence suggests that NMDA receptors are important sites of action of alcohols in the central nervous system, but studies performed to date have not identified the molecular mechanism by which alcohols modulate NMDA receptor function. Alcohol inhibition of NMDA receptors is noncompetitive with respect to the agonist (Göthert and Fink, 1989; Gonzales and Woodward, 1990; Rabe and Tabakoff, 1990; Peoples et al., 1997). 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), and results from a study in rat cerebellar granule neurons suggest that the apparent interaction of ethanol with the glycine site results instead from an action of alcohol on an unidentified intracellular modulator (Popp et al., 1999). It thus seems most probable that the glycine site may regulate or influence alcohol sensitivity under certain condi-

TABLE 1

Apparent change in energy of binding of diols and alcohols due to addition of a methylene group

Apparent ΔΔG values were determined as described under *Experimental Procedures*. Mean values did not differ significantly (Student's *t* test; $p > 0.05$).

No. of Carbon Atoms	Apparent ΔΔG due to addition of CH_2	
	1,Ω-Diols	1-Alcohols
	kJ/mol	
2		−1.28
3	−1.80	−2.92
4	−2.43	−2.31
5	−2.88	−1.76
6	−0.757	−1.93
7	−3.24	−1.55
8	−0.722	
9	−2.79	
Mean ± S.E.	−2.09 ± 0.387	−1.96 ± 0.240

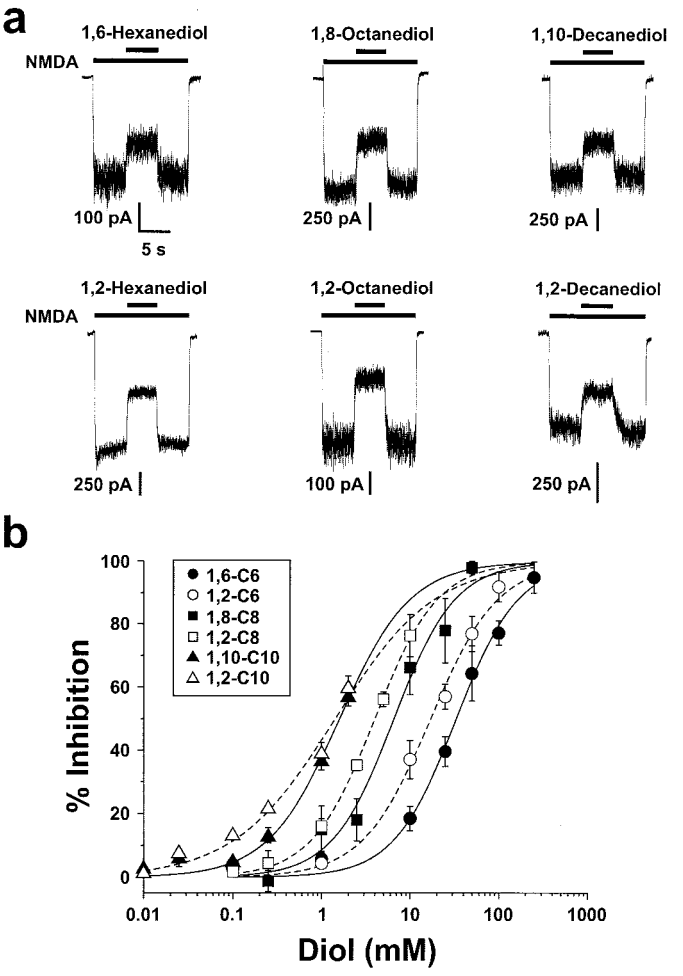

Fig. 5. Comparison of effects of 1,2-diols and 1,Ω-diols on NMDA receptors. a, records of current activated by 25 μM NMDA and 10 μM glycine and its inhibition by various 1,2- and 1,Ω-diols. Concentrations of diols were 25 mM 1,2- and 1,6-hexanediol, 10 mM 1,2- and 1,8-octanediol, and 1 mM 1,2- and 1,10-decanediol. Similar results were observed in four to six CHO cells tested. b, concentration-response curves for inhibition of current activated by 25 μM NMDA and 10 μM glycine by various 1,2- and 1,Ω-diols. Results are means of $n = 4$ to 8 cells. Curves shown are fits to equation 3 under *Experimental Procedures*. Respective IC_{50} and slope factor values were 17.1 ± 1.36 mM and 1.14 ± 0.111 for 1,2-hexanediol; 4.01 ± 0.0832 mM and 1.22 ± 0.0353 for 1,2-octanediol; and 1.44 ± 0.180 mM and 0.745 ± 0.0790 for 1,2-decanediol. IC_{50} values for 1,2-hexanediol and 1,2-octanediol differed significantly from those for the corresponding 1,Ω-diols (ALLFIT analysis; $p < 0.01$).

tions, but is not itself a site of alcohol action. The NMDA receptor also possesses a large number of sites for modulation by endogenous substances and drugs, many of which have been mapped to discrete domains on the receptor protein, but interactions of alcohols with these sites have not been observed (Chu et al., 1995; Peoples et al., 1997). In addition, alcohols do not seem to act via an open channel blocking mechanism (Wright et al., 1996).

In the present study, alcohols from ethanol to 1-nonanol inhibited NMDA-activated current in a concentration-dependent manner. Inhibitory potency of the alcohols increased with increases in carbon chain length up to 1-octanol. These results are similar to those obtained previously in mouse hippocampal neurons (Peoples and Weight, 1995) and in *Xenopus laevis* oocytes injected with mouse cerebral cortical mRNA (Dildy-Mayfield et al., 1996), except that the highest alcohol producing inhibition of NMDA receptors seemed to differ slightly among studies. In mouse hippocampal neurons, NMDA receptor inhibitory potency reached a peak at 1-heptanol, and maximal concentrations of 1-nonanol and higher alcohols did not inhibit NMDA receptors (Peoples and Weight, 1995). In contrast, in *X. laevis* oocytes injected with mouse cortex mRNA, the potency of alcohols for inhibition of NMDA receptors was not determined, but a maximal concentration of 1-decanol produced approximately 25% inhibition of the response to NMDA. The reasons for these discrepancies are not clear, but could involve differences in subunit composition of the NMDA receptors tested, as well as differences in experimental conditions. For example, in the present study, alcohols or diols were applied concurrently with agonist and intracellular Ca^{2+} was highly buffered. In contrast, in the study of Dildy-Mayfield et al. (1996), alcohols were applied for 30 s before exposure to agonist and intracellular

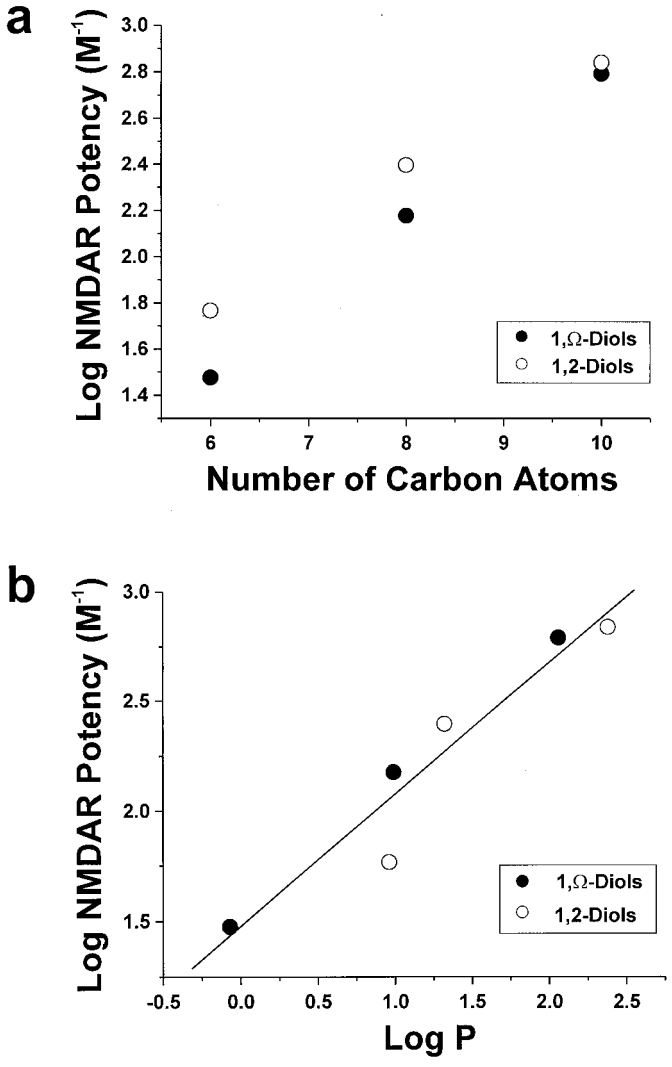

Fig. 6. Potency of 1,2- and 1,Ω-diols for inhibition of NMDA receptors. a, plot of NMDA receptor inhibitory potency ($1/IC_{50}$) versus carbon chain length. b, log NMDA receptor inhibitory potency ($1/IC_{50}$) versus hydrophobicity (log P) of 1,2- and 1,Ω-diols. The solid line is the least-squares fit to the data for both 1,2- and 1,Ω-diols ($y = 0.601x + 1.47$; $R^2 = 0.9587$; $p < 0.01$).

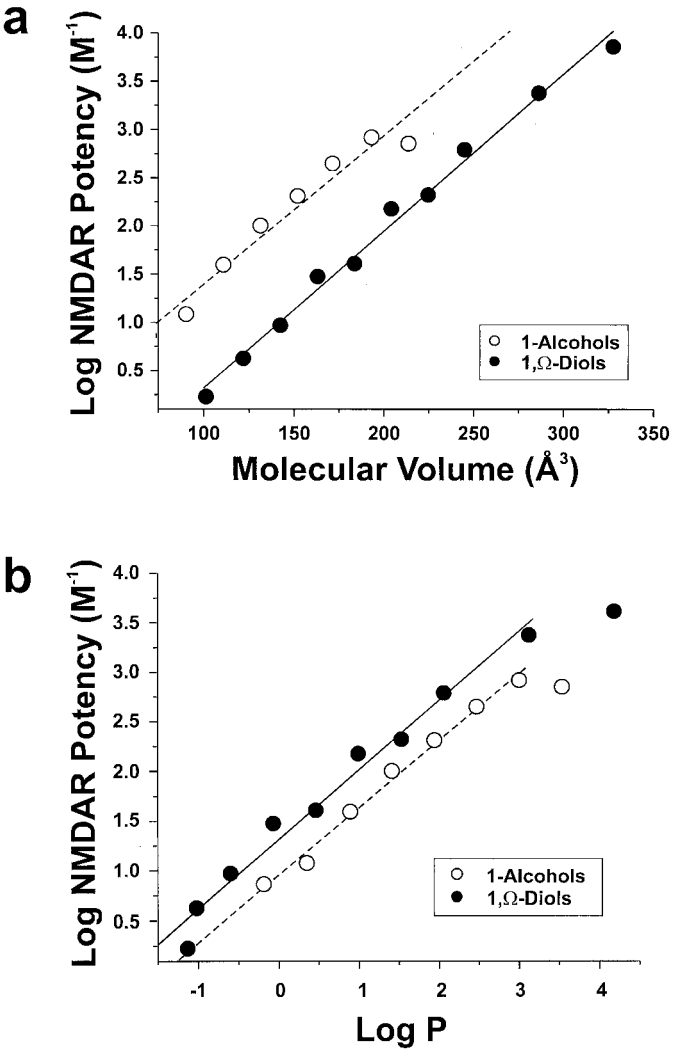

Fig. 7. Potency of alcohols and diols for inhibition of NMDA receptors as a function of physicochemical parameters. a, plot of NMDA receptor inhibitory potency ($1/IC_{50}$) versus molecular volume of alcohols and diols. The dashed line is the least-squares fit to the data for alcohols from ethanol to octanol ($y = 0.0154x - 0.151$; $R^2 = 0.9599$); the solid line is the least-squares fit to the data for diols from 1,3-propanediol to 1,12-dodecanediol ($y = 0.0163x - 1.31$; $R^2 = 0.9916$). Note that diols with molecular volumes exceeding that of nonanol, the largest alcohol producing inhibition of NMDA receptors, retain NMDA receptor inhibitory activity. b, log NMDA receptor inhibitory potency ($1/IC_{50}$) versus hydrophobicity (log P) of 1,Ω-diols and 1-alcohols. The dashed line is the least-squares fit to the data for alcohols from ethanol to octanol ($y = 0.680x + 0.956$; $R^2 = 0.9941$); the solid line is the least-squares fit to the data for diols from 1,3-propanediol to 1,12-dodecanediol ($y = 0.700x + 1.32$; $R^2 = 0.9889$).

Ca^{2+} was not buffered, which could increase the probability of interactions of alcohols with intracellular modulators resulting in increased NMDA receptor alcohol sensitivity (Popp et al., 1999).

In a previous study, single-channel recording and whole-cell noise analysis revealed that ethanol inhibition of NMDA receptors involves an equivalent reduction in the mean open time and frequency of opening of the ion channels, without any alteration in the unitary conductance (Wright et al., 1996). In the present study, fluctuation analysis revealed that 1,6-hexanediol produced similar effects on NMDA receptors. A concentration of hexanediol that inhibited NMDA-activated current by 39% produced a 23% reduction in the mean open time of the NMDA receptor channel but did not alter the unitary conductance of the channel; this is in good agreement with the observation by Wright et al. (1996) that a concentration of ethanol that inhibited NMDA-activated current by 35 to 40% reduced the mean open time of the channel by 23 to 26%. Because the mechanism by which alcohols inhibit NMDA receptors has not been established at present, it is not possible to demonstrate conclusively that straight-chain diols and alcohols inhibit NMDA receptors via a common mechanism. In light of the structural similarities of the two classes of molecules, as well as the similarities in their effects on NMDA receptors, however, it seems highly plausible that both classes of compounds inhibit NMDA receptors by acting at a common site.

Diols from 1,3-propanediol to 1,14-tetradecanediol inhibited NMDA-activated current in a concentration-dependent manner in the present study. As was observed for the series of alcohols tested, the inhibitory potency of the diols increased with increases in carbon chain length, and the average change in apparent energy of binding ($\Delta\Delta G$) due to the addition of a methylene group (below the cutoff point) was in the range expected for a hydrophobic interaction with a protein (Nozaki and Tanford, 1971). Furthermore, this value, -2.09 ± 0.387 kJ/mol, did not differ significantly from the average values of $\Delta\Delta G$ due to addition of a methylene group to a series of *n*-alcohols below the cutoff point for inhibition of either NMDA receptors in mouse hippocampal neurons in a previous study (Peoples and Weight, 1995) or NR1/NR2B NMDA receptors in CHO cells in the present study (-1.92 ± 0.313 and -1.96 ± 0.240 kJ/mol, respectively; ANOVA; $p > 0.05$). These observations are consistent with a common site of action of alcohols and diols on the NMDA receptor, as well as with an important role of hydrophobic interactions in the association of the alcohols and diols with their site of action on the NMDA receptor.

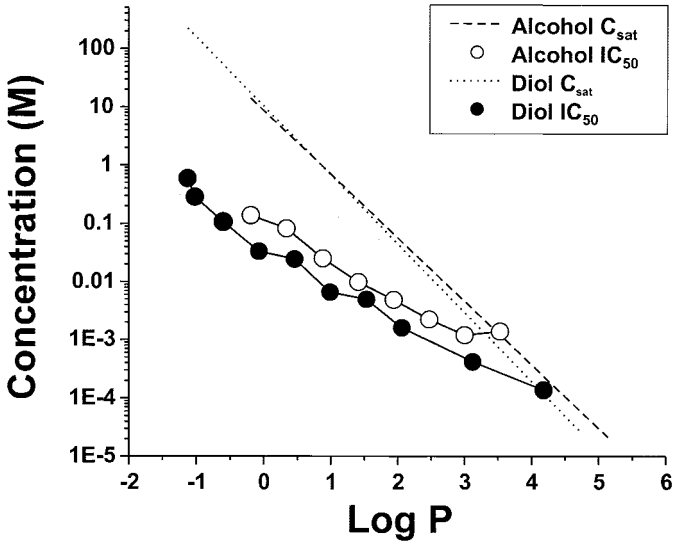

Fig. 8. Comparison of the potency of alcohols and diols for inhibition of NMDA receptors with their saturating aqueous concentrations (C_{sat}). Graph plots NMDA receptor inhibitory potency ($1/IC_{50}$) versus log P of alcohols and diols. The dashed line is the least-squares fit to the C_{sat} for alcohols obtained from Bell (1973); the dotted line is the least-squares fit to the C_{sat} for diols, calculated as described under *Experimental Procedures*.

The position of the second hydroxyl group along the carbon chain of the diols also seemed to influence their effects on the NMDA receptor. When the additional hydroxyl group was located on the second carbon atom in hexanediol and octanediol, the resulting compounds were more potent in inhibiting NMDA receptors than were the corresponding 1,Ω-diols. This effect apparently resulted primarily from the increased hydrophobicity of the 1,2- versus the 1,Ω-diols, however, as was evident from a plot of NMDA receptor potency versus log P for these diols.

In a previous study, it was proposed that the observed cutoff in alcohol inhibition of NMDA receptors might be due to the exclusion of larger alcohols from a binding site of fixed dimensions on the NMDA receptor protein (Peoples and Weight, 1995). The observation of the present study, however, that the NMDA receptor inhibitory potency of diols continued to increase well beyond the molecular volume of 1-nonanol (213.8 $Å^3$), is not consistent with this interpretation. Thus, if the assumption that alcohols and diols act at a common site is correct, the molecular volumes of the alcohols near the cutoff point cannot be used to draw inferences about the dimensions of the site of alcohol action on the NMDA receptor protein. A similar lack of dependence on molecular volume of the cutoff phenomenon for alcohol interaction with the soluble protein bovine serum albumin has been reported by Eckenhoff et al. (1999). These findings, taken together with results of studies on other neurotransmitter-gated ion channels demonstrating dependence of alcohol and anesthetic interactions upon molecular volume (Wick et al., 1998; Jenkins et al., 2001), underscore the point that the physical basis of the cutoff phenomenon may vary among proteins (Peoples et al., 1996), and that observations of cutoff per se do not constitute direct evidence of specific interactions or steric hindrance. The observation of the present study that diols were slightly more potent than alcohols at equivalent hydrophobicity may indicate that the additional hydroxyl group present on the diols contributed to the binding to the site of action, which is consistent with previous reports that binding sites of alcohols and anesthetics possess both hydrophobic and hydrogen bonding characteristics (Abraham et al., 1991). For both alcohols and diols, the slope of the line obtained from a plot of the NMDA receptor inhibitory potency versus the number of carbon atoms yielded an approximate prediction of the cutoff point (Fig. 8). This suggests that the cutoff resulted largely because this line was not parallel to the line describing the relationship between the maximum aqueous solubility and the number of carbon atoms of the alcohols or diols. In other words, the addition of a methylene group to the alcohols or diols produced a decrease in the aqueous solubility that exceeded the increase in binding energy to their site

of action, and the cutoff resulted when it was no longer possible to achieve an aqueous concentration above the threshold for receptor inhibition. The curvature of the plot near the cutoff point, however, may indicate a contribution of additional factors to the cutoff phenomenon.

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References

Abraham MH, Lieb WR, and Franks NP (1991) Role of hydrogen bonding in general anesthesia. *J Pharmacol Sci* **80:**719–724.

Bell GH (1973) Solubilities of normal aliphatic acids, alcohols and alkanes in water. *Chem Phys Lipids* **10:**1–10.

Buller AL, Larson HC, Morrisett RA, and Monaghan DT (1995) Glycine modulates ethanol inhibition of heteromeric *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *Mol Pharmacol* **48:**717–723.

Cebers G, Cebere A, Zharkovsky A, and Liljequist S (1996) Glycine does not reverse the inhibitory actions of ethanol on NMDA receptor functions in cerebellar granule cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **354:**736–745.

Chiou J-S, Ma S-M, Kamaya H, and Ueda I (1990) Anesthesia cutoff phenomenon: interfacial hydrogen bonding. *Science (Wash DC)* **248:**583–585.

Chu B, Anantharam V, and Treistman SN (1995) Ethanol inhibition of recombinant heteromeric NMDA channels in the presence and absence of modulators. *J Neurochem* **65:**140–148.

DeLean A, Munson PJ, and Rodbard D (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am J Physiol* **235:**E97–E102.

Dildy-Mayfield JE and Leslie SW (1991) Mechanism of inhibition of *N*-methyl-D-aspartate-stimulated increases in free intracellular Ca^{2+} concentration by ethanol. *J Neurochemistry* **56:**1536–1543.

Dildy-Mayfield JE, Mihic SJ, Liu Y, Deitrich RA, and Harris RA (1996) Actions of long chain alcohols on $GABA_a$ and glutamate receptors: relation to *in vivo* effects. *Br J Pharmacol* **118:**378–384.

Eckenhoff RG, Tanner JW, and Johansson JS (1999) Steric hindrance is not required for *n*-alkanol cutoff in soluble proteins. *Mol Pharmacol* **56:**414–418.

Franks NP and Lieb WR (1985) Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects. *Nature (Lond)* **316:**349–351.

Gonzales RA and Woodward JJ (1990) Ethanol inhibits *N*-methyl-D-aspartate-stimulated [^{3}H]norepinephrine release from rat cortical slices. *J Pharmacol Exp Ther* **253:**1138–1144.

Göthert M and Fink K (1989) Inhibition of *N*-methyl-D-aspartate (NMDA)- and l-glutamate-induced noradrenaline and acetylcholine release in the rat brain by ethanol. *Naunyn-Schmiedeberg's Arch Pharmacol* **340:**516–521.

Hoffman PL, Rabe CS, Moses F, and Tabakoff B (1989) *N*-Methyl-D-aspartate receptors and ethanol: inhibition of calcium flux and cyclic GMP production. *J Neurochem* **52:**1937–1940.

Jenkins A, Greenblatt EP, Faulkner HJ, Bertaccini E, Light A, Lin A, Andreasen A, Viner A, Trudell JR, and Harrison NL (2001) Evidence for a common binding cavity for three general anesthetics within the $GABA_A$ receptor. *J Neurosci* **21:**RC136:1–4.

Lee AG (1976) Interactions between anesthetics and lipid mixtures. *Normal Alcohols Biochem* **15:**2448–2454.

Li C, Peoples RW, and Weight FF (1994) Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein. *Proc Natl Acad Sci USA* **91:**8200–8204.

Li CY, Peoples RW, and Weight FF (1998) Ethanol-induced inhibition of a neuronal P2X purinoceptor by an allosteric mechanism. *Br J Pharmacol* **123:**1–3.

Lyon RC, McComb JA, Schreurs J, and Goldstein DB (1981) A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. *J Pharmacol Exp Ther* **218:**669–675.

Mascia MP, Machu TK, and Harris RA (1996) Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br J Pharmacol* **119:**1331–1336.

Mirshahi T and Woodward JJ (1995) Ethanol sensitivity of heteromeric NMDA receptors: effects of subunit assembly, glycine and NMDAR1 Mg^{2+}-insensitive mutants. *Neuropharmacology* **34:**347–355.

Mitchell DC, Lawrence JTR, and Litman BJ (1996) Primary alcohols modulate the activation of the G protein-coupled receptor rhodopsin by a lipid-mediated mechanism. *J Biol Chem* **271:**19033–19036.

Moody EJ, Yeh HJC, and Skolnick P (1991) The $GABA_A$ receptor complex: is it a locus of action for inhalation anesthetics? in *Neuropharmacology of Ethanol: New Approaches* (Meyer RE, Koob GF, Lewis MJ, and Paul SM eds) pp 77–92, Birkhäuser, Boston.

Murrell RD, Braun MS, and Haydon DA (1991) Actions of *n*-alcohols on nicotinic acetylcholine receptor channels in cultured rat myotubes. *J Physiol (Lond)* **437:**431–448.

Nozaki Y and Tanford C (1971) The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. *J Biol Chem* **246:**2211–2217.

Peoples RW, Li C, and Weight FF (1996) Lipid vs protein theories of alcohol action in the nervous system. *Annu Rev Pharmacol Toxicol* **36:**185–201.

Peoples RW and Stewart RR (2000) Alcohols inhibit *N*-methyl-D-aspartate receptors via a site exposed to the extracellular environment. *Neuropharmacology* **39:**1681–1691.

Peoples RW and Weight FF (1992) Ethanol inhibition of *N*-methyl-D-aspartate-activated ion current in rat hippocampal neurons is not competitive with glycine. *Brain Res* **571:**342–344.

Peoples RW and Weight FF (1995) Cutoff in potency implicates alcohol inhibition of *N*-methyl-D-aspartate receptors in alcohol intoxication. *Proc Natl Acad Sci USA* **92:**2825–2829.

Peoples RW, White G, Lovinger DM, and Weight FF (1997) Ethanol inhibition of *N*-methyl-D-aspartate-activated current in mouse hippocampal neurones: whole-cell patch-clamp analysis. *Br J Pharmacol* **122:**1035–1042.

Popp, Lickteig RL, and Lovinger DM (1999) Factors that enhance ethanol inhibition of *N*-methyl-D-aspartate receptors in cerebellar granule cells. *J Pharmacol Exp Ther* **289:**1564–1574.

Rabe CS and Tabakoff B (1990) Glycine site-directed agonists reverse the actions of ethanol at the *N*-methyl-D-aspartate receptor. *Mol Pharmacol* **38:**753–757.

Wick MJ, Mihic SJ, Ueno S, Mascia MP, Trudell JR, Brozowski SJ, Ye Q, Harrison NL, and Harris RA (1998) Mutations of γ-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor? *Proc Natl Acad Sci USA* **95:**6504–6509.

Woodward JJ (1994) A comparison of the effects of ethanol and the competitive glycine antagonist 7-chlorokynurenic acid on *N*-methyl-D-aspartic acid-induced neurotransmitter release from rat hippocampal slices. *J Neurochem* **62:**987–991.

Woodward JJ and Gonzales RA (1990) Ethanol inhibition of *N*-methyl-D-aspartate-stimulated endogenous dopamine release from rat striatal slices: reversal by glycine. *J Neurochem* **54:**712–715.

Wright JM, Peoples RW, and Weight FF (1996) Single-channel and whole-cell analysis of ethanol inhibition of NMDA-activated currents in cultured mouse cortical and hippocampal neurons. *Brain Res* **738:**249–256.

Zilberter Y, Uteshev V, Sokolova S, and Khodorov B (1991) Desensitization of *N*-methyl-D-aspartate receptors in neurons dissociated from adult rat hippocampus. *Mol Pharmacol* **40:**337–341.

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