British Journal of Pharmacology (1997) 122, 1035–1042

Ethanol inhibition of *N*-methyl-D-aspartate-activated current in mouse hippocampal neurones: whole-cell patch-clamp analysis

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1 The action of ethanol on *N*-methyl-D-aspartate (NMDA)-activated ion current was studied in mouse hippocampal neurones in culture using whole-cell patch-clamp recording.

2 Ethanol inhibited NMDA-activated current in a voltage-independent manner, and did not alter the reversal potential of NMDA-activated current.

3 Concentration–response analysis of NMDA- and glycine-activated current revealed that ethanol decreased the maximal response to both agonists without affecting their EC_{50} values.

4 The polyamine spermine (1 μM) increased amplitude of NMDA-activated current but did not alter the percentage inhibition of ethanol.

5 Compared to an extracellular pH of 7.0, pH 6.0 decreased and pH 8.0 increased the amplitude of NMDA-activated current, but these changes in pH did not significantly alter the percentage inhibition by ethanol.

6 The sulphydryl reducing agent dithiothreitol (2 mM) increased the amplitude of NMDA-activated current, but did not affect the percentage inhibition by ethanol.

7 Mg^{2+} (10, 100, 500 μM), Zn^{2+} (5, 20 μM) or ketamine (2, 10 μM) decreased the amplitude of NMDA-activated current, but did not affect the percentage inhibition by ethanol.

8 The observations are consistent with ethanol inhibiting the function of NMDA receptors by a non-competitive mechanism that does not involve several modulatory sites on the NMDA receptor–ionophore complex.

Keywords: NMDA; ethanol; hippocampus; glycine; Mg^{2+}; Zn^{2+}; ketamine; polyamine; proton; oxidation-reduction

Introduction

Ethanol, at concentrations that induce intoxication in non-tolerant animals (5–100 mM), has been found to inhibit many NMDA receptor-mediated responses in neurones. Ethanol inhibits NMDA-activated ion current in cultured hippocampal neurones (Lovinger *et al*., 1989) and in freshly isolated sensory neurones (White *et al*., 1990b), and NMDA receptor-mediated excitatory synaptic transmission in hippocampal slices (Lovinger *et al*., 1990b). Similarly, ethanol inhibits NMDA-stimulated $^{45}Ca^{2+}$ uptake and cyclic GMP production (Hoffman *et al*., 1989), and NMDA-induced neurotransmitter release from brain slices (Göthert & Fink, 1989; Woodward & Gonzales, 1990).

The mechanism by which ethanol inhibits NMDA receptor-activated responses is not known. Several studies have reported that in some instances the inhibitory action of ethanol is reduced at high concentrations of glycine (Hoffman *et al*., 1989; Rabe & Tabakoff, 1990; Woodward & Gonzales, 1990; Dildy-Mayfield & Leslie, 1991), which is a coagonist at the NMDA receptor/channel complex (Johnson & Ascher, 1987; Kleckner & Dingledine, 1988), although in other experiments no such effect of glycine was observed (Gonzales & Woodward, 1990; Peoples & Weight, 1992; Woodward, 1994b); Chu *et al*., 1995; Mirshahi & Woodward, 1995; Cebers *et al*., 1996). In addition, dependence of ethanol inhibition of NMDA receptor-mediated responses on the concentration of Mg^{2+} (which blocks NMDA receptor channels) in hippocampal slices has been reported (Martin *et al*., 1991; Morrisett *et al*., 1991). An initial study suggested that ethanol does not interact with the NMDA/glutamate binding site or the phencyclidine binding site on the receptor channel complex (Rabe & Tabakoff, 1990); however, a subsequent study suggested such an interaction (Dildy-Mayfield & Leslie, 1991). We have investigated the interaction between ethanol and various modulatory sites on the NMDA receptor complex using whole-cell patch-clamp recording in hippocampal neurones in culture. Use of this recording technique has also permitted examination of whether the action of ethanol is voltage-dependent, and determination of whether ethanol alters the reversal potential of NMDA-activated current. Some of these results have been reported previously in preliminary form (Lovinger *et al*., 1990a; White *et al*., 1990a; Peoples & Weight, 1991).

Methods

Experiments were carried out using hippocampal neurones obtained from foetal mice and maintained in cell culture as described by Forsythe & Westbrook (1988). Electrophysiological recording of NMDA-activated current was studied with the whole-cell patch-clamp technique using the EPC-7 (List Medical) or the Axopatch 1D (Axon Instruments) patch-clamp amplifier essentially as described previously (Lovinger *et al*., 1989). The extracellular recording medium contained (in mM): 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 10 HEPES, 10 D-glucose, 0.0002 tetrodotoxin; pH was adjusted to 7.4 using NaOH. The patch-pipette (intracellular) solution contained (in mM): 140 CsCl, 1 $CaCl_2$, 2 $MgCl_2$ 10 BAPTA, 10 HEPES, 2 Mg_4ATP; pH was adjusted to 7.4 using CsOH. Recording pipette tip resistances

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were 2–5 MΩ. Series resistances of 3–10 MΩ were compensated by 40–80%. All neurones were voltage-clamped at −50 mV, unless otherwise indicated.

Data were displayed on a digital oscilloscope (Nicolet 1090-111A) and recorded using a chart recorder (Gould 2400S). In some cases data were also recorded on a computer (Compaq 386/20e) using a LabMaster TL-125 interface and pCLAMP or AXOTAPE software (Axon Instruments).

Drug solutions were applied to neurones by gravity flow using one of two methods. In some experiments, a linear multi-barrel pipette array (diameter of each pipette ~200 μm) was placed within 100 μm of the cell body. Cells were constantly superfused with an extracellular medium flowing from one barrel (flow rate ~3 μl s^{-1}), and drug solutions were applied by opening a valve connected to another barrel and moving the barrel array so that the desired solution superfused the cell. In the other experiments, drug solutions were applied by lowering a large bore (200–300 μm tip diameter) pipette to within 30 μm of the neuronal soma. Unless stated otherwise, NMDA solutions also contained 200 nM glycine. Agonists were applied at intervals of at least 90 s to allow for recovery from receptor desensitisation.

Statistical analysis of concentration–response data was performed using the nonlinear curve-fitting program ALLFIT (DeLean *et al*., 1978). Values reported from concentration–response analysis are those obtained by fitting the data to the equation

$$y = \frac{E_{max} - E_{min}}{1 + (x/EC_{50})^{-n}} + E_{min}$$

where x and y are concentration and response, respectively, E_{min} is the minimal response, E_{max} is the maximal response, EC_{50} is the half-maximal concentration, and n is the slope factor. Statistical comparisons were performed using paired Student's *t* tests or analysis of variance (ANOVA), as indicated. Average values are reported as the mean ± s.e.mean.

Ethanol (95%; prepared from grain) was purchased from Pharmco, tetrodotoxin from Calbiochem, and salts from Mallinkrodt. All other chemicals were purchased from Sigma Chemical.

The care and use of animals in this study was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism in accordance with National Institutes of Health guidelines (protocol number: LMCN-SP-01).

Results

Previous results obtained in this laboratory have demonstrated that ethanol inhibits NMDA-activated current in mouse hippocampal neurons in culture (Lovinger *et al*., 1989). Sensitivity of the NMDA receptor-channel to ethanol may vary somewhat depending upon culture conditions. Under culture conditions similar to those used in these experiments, ethanol inhibited NMDA-activated current with an IC_{50} of ~130 mM and a maximal response of 100% inhibition (Peoples & Weight, 1995). Although in some cases ethanol inhibited NMDA-activated current more potently, results of most of the experiments reported here were consistent with these values.

Influence of membrane potential on ethanol inhibition of NMDA-activated current

To determine whether ethanol inhibition of NMDA-activated current is voltage-dependent, we tested the inhibitory effect of ethanol at membrane holding potentials from +60 to −60 mV using a voltage-ramp stimulus (negative-going; slew rate: 125 mV s^{-1}). Figure 1a shows records of the inward current activated by 50 μM NMDA and its inhibition by 50 mM ethanol in a typical neurone; the large deflections in the current traces resulted from application of the voltage-ramp stimulus. A plot of the current–voltage relationship for NMDA-activated current in the absence and the presence of ethanol during the voltage-ramp stimulus is illustrated in Figure 1b. In this cell, ethanol inhibition of NMDA-activated

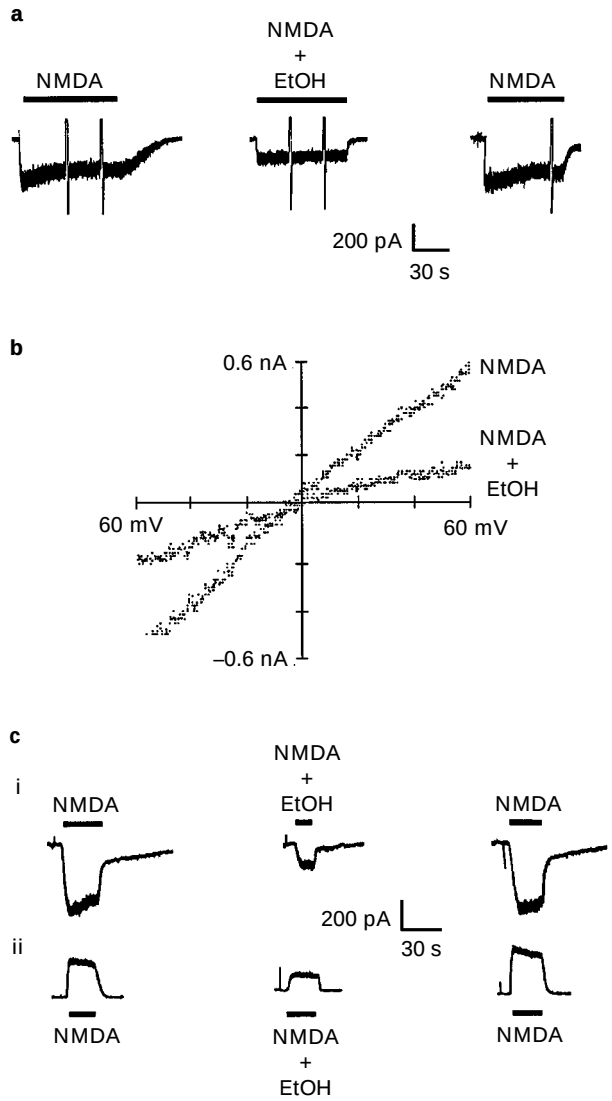

Figure 1 Ethanol inhibition of NMDA-activated current is not voltage-dependent. (a) Current activated by NMDA, 50 μM, in the absence and the presence of 50 mM ethanol (EtOH). Application of a voltage ramp stimulus (described below) produced the large deflections evident in the current traces. Membrane potential was held at −50 mV, except during the ramp stimulus. Solid bar above each record indicates time of agonist and/or drug application, as labelled. (b) Plot of NMDA-activated current as a function of membrane potential in the absence (NMDA) and presence (NMDA + EtOH) of 50 mM EtOH. Membrane potential was rapidly changed using a voltage ramp stimulus (−50 to +60 mV, +60 to −60 mV, −60 to −50 mV; slew rate: 125 mV s^{-1}). Data were acquired during the negative-going phase of the voltage ramp stimulus to minimise effects of voltage-activated ion channels. Current obtained using the same stimulus in the absence of NMDA was subtracted from the current in the presence of NMDA. In some neurones the stimulus was applied from a more depolarized holding potential in order to decrease the amplitude of the voltage-activated current through time-dependent (on the order of minutes) inactivation. Data in (b) are from the records shown in (a). (c) Records of NMDA-activated current at membrane potentials of −50 (i) and +30 mV (ii) in the absence and the presence of 50 mM ethanol (EtOH). Membrane potential was held at −50 or +30 mV for at least 3 min before evaluating the effect of 50 μM ethanol on amplitude of NMDA-activated ion current. Solid bar above each record indicates time of agonist and/or drug application, as labelled.

current was not voltage-dependent, and ethanol did not appear to change the reversal potential of the current. On average, the percentage inhibition of NMDA-activated current by ethanol at a membrane potential of +60 mV did not differ from that at a membrane potential of −60 mV (59±5 vs 62±3%, respectively; $P>0.1$). In addition, ethanol did not alter the average reversal potential of the current (0±6 mV in the absence of ethanol vs 2±5 mV in the presence of ethanol; $P>0.1$). Because voltage-dependence could have a time-dependent component which is slower than the slew rate of the voltage-ramp (Nowak & Wright, 1992), we also studied the effect of ethanol while holding membrane potential constant for >3 min (Figure 1c). The percentage reduction in amplitude of NMDA-activated current by ethanol under these conditions was not significantly different at membrane potentials of −50 mV and +30 mV (44±9 and 44±7% respectively, $P>0.1$).

Effect of ethanol on the NMDA concentration–response

Ethanol could inhibit the NMDA receptor competitively by displacing the agonist from the NMDA/glutamate binding site. To assess this possibility, we constructed concentration–response curves for NMDA in the absence and the presence of ethanol. Figure 2a shows records of currents activated by two concentrations of NMDA and their inhibition by ethanol. In this neurone, 100 mM ethanol inhibited current activated by 25 μM NMDA by 32%, and inhibited current activated by 250 μM NMDA by 27%. Figure 2b shows average NMDA-activated current plotted as a function of NMDA concentration over the range 1 to 1000 μM in the absence and presence of 100 mM ethanol. Ethanol did not significantly alter the EC_{50} of NMDA (32.6 μM in the absence of ethanol vs 41.0 μM in the presence of ethanol; analysis of variance, $P>0.1$), but decreased the maximal response to NMDA by 23% (analysis of variance, $P<0.05$).

Effect of ethanol on the glycine concentration–response

It has been suggested that ethanol may inhibit the NMDA receptor by decreasing the potency of the coagonist glycine (Hoffman *et al*., 1989). Ethanol could produce this effect either by competing with glycine for its binding site or by acting allosterically to reduce the affinity of the site for glycine. To determine whether ethanol altered the potency of glycine, we performed a concentration–response analysis for glycine in the absence and the presence of ethanol. The graph in Figure 3 shows normalized current amplitude plotted as a function of glycine concentration over the range 100 nM to 10.1 μM in the absence and presence of 50 mM ethanol. Ethanol did not significantly alter the EC_{50} of glycine (227 nM in the absence of ethanol vs 273 nM in the presence of ethanol; analysis of variance, $P>0.05$), but decreased the maximal response to glycine (222% of response to 200 nM glycine in the absence of ethanol vs 171% of response to 200 nM glycine in the presence of ethanol; ANOVA, $P<0.01$).

Effect of the polyamine spermine on ethanol inhibition of NMDA-activated current

Polyamines can act via multiple mechanisms to either potentiate or inhibit NMDA receptor-mediated responses, depending upon the subunit composition of the receptor-channels (Rock & Macdonald, 1995; Johnson, 1996). In the neurones used in the present study, polyamines at low concentrations enhanced NMDA-activated current. We investigated a possible interaction of ethanol with this site by testing whether the effect of ethanol was altered in the presence of the polyamine spermine. Figure 4a show traces of current activated by NMDA in the absence and the presence of spermine, and their inhibition by ethanol in a hippocampal neurone. Spermine, 1 μM, enhanced NMDA-activated current in this cell, but had little apparent effect on ethanol inhibition. On

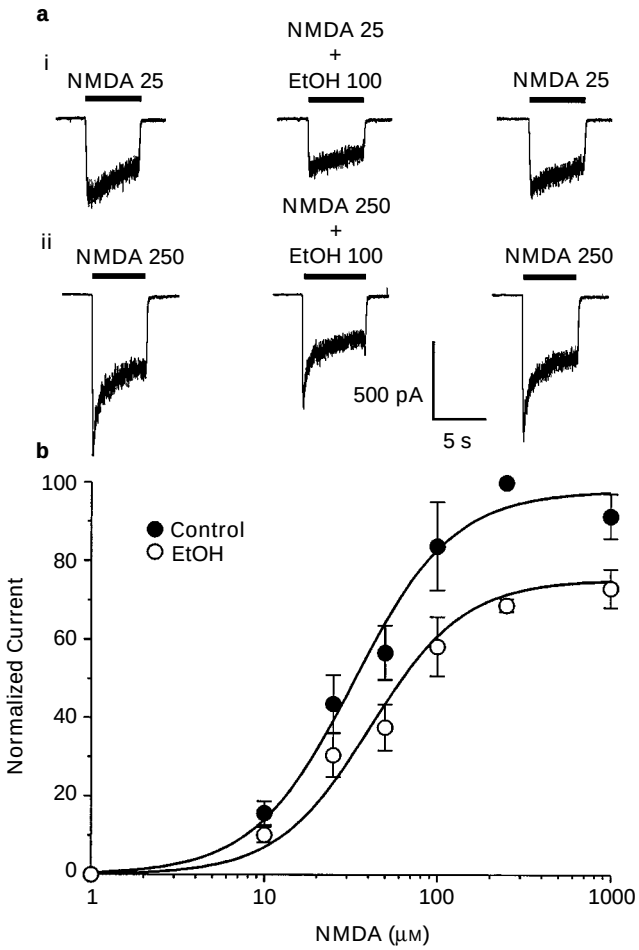

Figure 2 Ethanol inhibition of NMDA-activated current is not competitive with NMDA. (a) Records of current activated by 25 μM (i) and 250 μM (ii) NMDA and the inhibition of those currents by 100 mM ethanol (EtOH) in a hippocampal neurone. Solid bar above each record indicates time of agonist and/or drug application, as labelled. Time and current calibrations in (i) apply to all records. (b) The graph plots the percentage of current activated by 250 μM NMDA as a function of NMDA concentration in the absence (●) and presence (○) of 100 mM ethanol. Solutions of NMDA also contained 10 μM glycine. Each data point is the mean±s.e.mean of 6–7 neurones. The curves shown are the best fits of the data to the equation described in the Methods.

average, 1 μM spermine potentiated NMDA-activated current by 18±4%, but did not alter the percentage inhibition by ethanol of NMDA-activated current (Figure 4b; 32±5% in the absence of spermine (control) vs 34±3% inhibition in the presence of spermine; $P>0.1$).

Effect of pH on ethanol inhibition of NMDA-activated current

The NMDA receptor has been observed to be sensitive to extracellular pH: increasing extracellular pH potentiates NMDA-activated current, while decreasing pH inhibits NMDA-activated current (Traynelis & Cull-Candy, 1990; Vyklicky *et al*., 1990). We examined whether ethanol interacts with the proton modulatory site of the NMDA receptor by determining the extent of inhibition by ethanol when the extracellular pH was buffered at 6.0, 7.0 or 8.0. Results from a typical neurone are shown in Figure 5a. In this cell, amplitude of current activated by NMDA was highly dependent upon pH (55 pA at pH 6, 370 pA at pH 7, and 950 pA at pH 8), but pH had little effect on ethanol inhibition. The average percentage inhibition by ethanol was not significantly different at the different pH values (Figure 5b); the average inhibition by

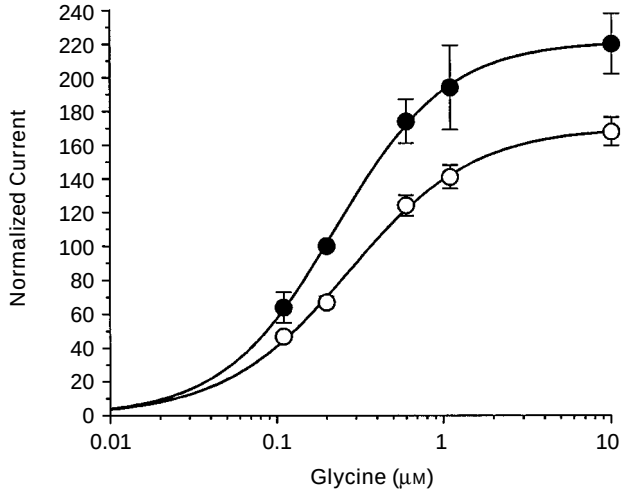

Figure 3 Ethanol inhibition of NMDA-activated current is not competitive with glycine. The graph plots the percentage of current activated by 50 μM NMDA as a function of glycine concentration in the absence (●) and presence (○) of 50 mM ethanol. Each data point is the mean ± s.e.mean of at least 7 neurones. The curves shown are the best fits of the data to the equation described in the Methods.

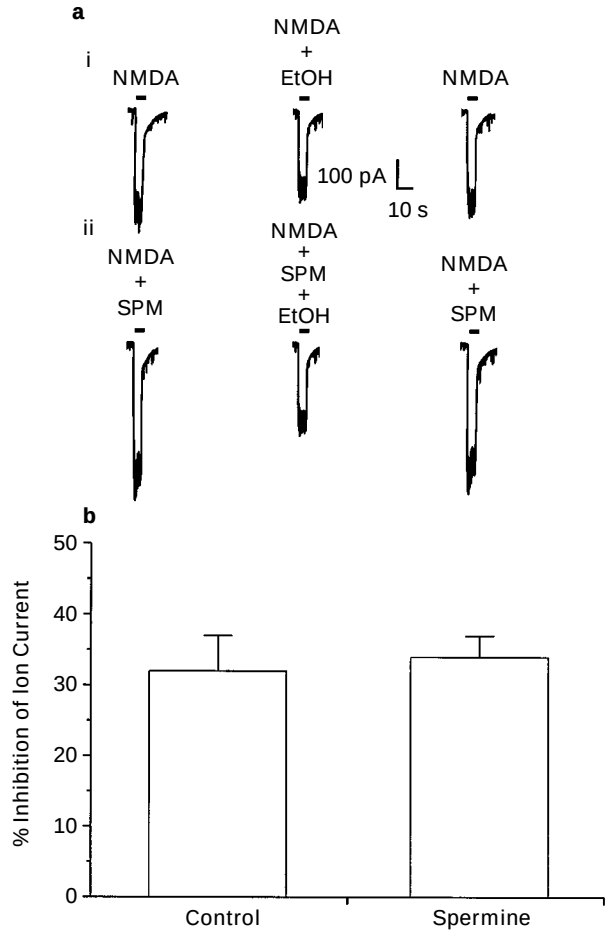

Figure 4 Ethanol inhibition of NMDA-activated current is not reduced in the presence of the polyamine spermine. (a) Records of inward current activated by 25 μM NMDA and its inhibition by 100 mM ethanol (EtOH) in the absence (i) and the presence (ii) of 1 μM spermine (SPM). All records are from the same neurone; time and current calibrations in (i) apply to all records. (b) Comparison of average effect of 100 mM ethanol (EtOH) on current activated by 25 μM NMDA, plotted as percentage inhibition of NMDA-activated current, in the absence and presence of 1 μM spermine, as labelled. Results are means ± s.e.mean of 6 neurones.

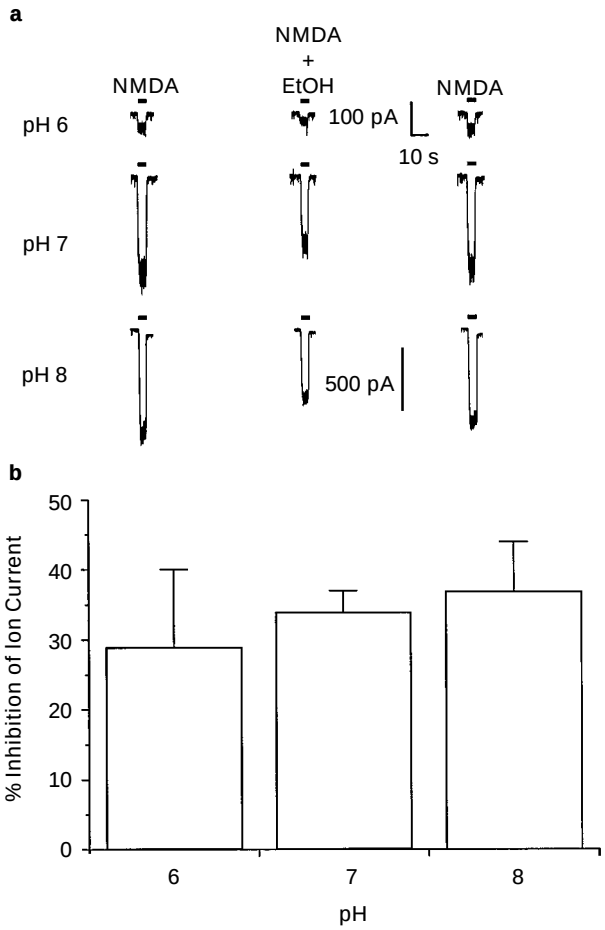

Figure 5 Ethanol inhibition of NMDA-activated current is independent of extracellular pH. (a) Records of inward current activated by 25 μM NMDA and its inhibition by 100 mM ethanol (EtOH) at pH 6.0, 7.0 and 8.0. All records are from the same neurone. The neurone was allowed to adjust to changes in pH for at least 1 min before applying NMDA. Solid bar above each record indicates time of agonist and/or drug application, as labelled. Time calibration in pH 6.0 applies to all records; current calibration in pH 6.0 also applies to records in pH 7.0. (b) Comparison of average effect of 100 mM ethanol (EtOH) on current activated by 25 μM NMDA and 200 nM glycine, plotted as percentage inhibition of NMDA-activated current, at pH 6.0, 7.0 and 8.0. Results are means ± s.e.mean of 4 neurones.

100 mM ethanol was 29 ± 11% at pH 6, 34 ± 3% at pH 7 and 37 ± 7% at pH 8 (repeated-measures analysis of variance, $P > 0.1$).

Effect of oxidation and reduction on ethanol inhibition of NMDA-activated current

Reduction of disulphide groups by agents such as dithiothreitol (DTT) can modulate the activity of the NMDA receptor channel by increasing the frequency of opening with little or no change in the mean open time (Tang & Aizenman, 1993). This potentiation can be reversed by the oxidizing agent 5-5-dithio-bis-2-nitrobenzoic acid (DTNB), which can also slightly attenuate the response in some preparations prior to treatment by DTT. In the present study, a 2 min application of 2 mM DTT to previously untreated neurones enhanced current amplitude (172 ± 17% of control amplitude; $P < 0.05$), whereas a similar treatment with DTNB did not significantly affect NMDA-activated current (84 ± 14% of control amplitude; $P > 0.05$). This suggests that the sulphydryl groups constituting the oxidation-reduction (redox) modulatory site existed primarily in the oxidized state prior to treatment. These results

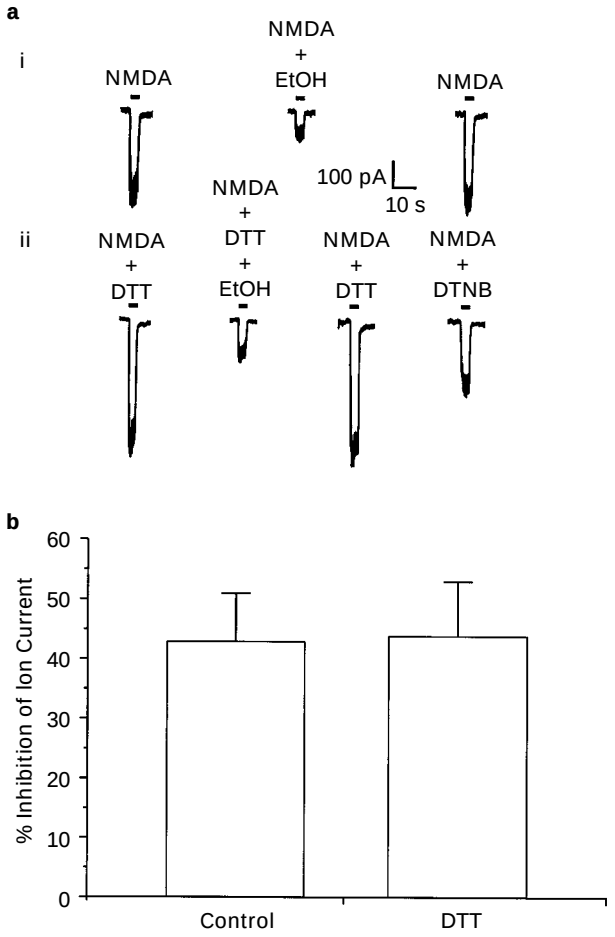

Figure 6 Ethanol inhibition is not altered by changes in the oxidation/reduction state of the receptor. (a) Records of inward current activated by 25 μM NMDA and its inhibition by 100 mM ethanol (EtOH) preceding (i) and following (ii) a 2 min exposure to 2 mM dithiothreitol (DTT). A 2 min exposure to 2 mM 5-5-dithio-bis-2-nitrobenzoic acid (DTNB) reversed the enhancement of NMDA-activated current by DTT, as shown by the current trace at the far right. All records are from the same neuron; time and current calibrations in (i) apply to all records. (b) Comparison of average effect of 100 mM ethanol (EtOH) on current activated by 25 μM NMDA, plotted as percentage inhibition of NMDA-activated current, preceding and following a 2 min exposure to 2 mM DTT, as labelled. Results are means$\pm$s.e.mean of 6 neurones.

are similar to the results reported by Tang & Aizenman (1993). We next evaluated whether ethanol might interact with this redox modulatory site. The records shown in Figure 6a illustrate the inhibition of NMDA-activated current both prior to (1) and following (2) DTT treatment. As shown in Figure 6b, the average percentage inhibition by 100 mM ethanol of NMDA-activated current under control conditions did not differ from that in the reduced state immediately following 2 min of DTT treatment (43 ± 8 vs 44 ± 9% inhibition, respectively; $P>0.1$).

Effect of Mg^{2+} on ethanol inhibition of NMDA-activated current

Previous studies have reported that Mg^{2+}, which blocks the NMDA-gated ion channel, increases the apparent potency of ethanol (Martin *et al*., 1991; Morrisett *et al*., 1991). Thus, Mg^{2+} binding to its site on the NMDA receptor may influence the site at which ethanol acts. We tested whether increasing Mg^{2+} concentration might increase the inhibition by ethanol. Table 1 shows that 50 mM ethanol produced a similar percentage inhibition of NMDA-activated current at concentrations of added Mg^{2+} from 0 to 500 μM (analysis of variance, $P>0.1$). In this experiment, 10 μM Mg^{2+} inhibited NMDA-activated current by less than 10% ($n=4$), while 100 and 500 μM Mg^{2+} inhibited NMDA-activated current by 47 ± 4% ($n=4$) and 69 ± 10% ($n=4$), respectively.

Effect of Zn^{2+} on ethanol inhibition of NMDA-activated current

Neuronal ion current activated by NMDA is also inhibited by Zn^{2+} acting at a site separate from that of Mg^{2+} (Mayer *et al*., 1988). We tested whether ethanol might interact with the Zn^{2+} modulatory site on the NMDA receptor–ionophore complex by studying the effect of ethanol at various concentrations of added Zn^{2+}. Table 1 shows the average percentage inhibition of NMDA-activated current by 50 mM ethanol in 0, 5 and 20 μM added Zn^{2+}. No significant differences were found among these groups (analysis of variance, $P>0.25$). The percentage inhibition by Zn^{2+} in the absence of ethanol was 39 ± 6% at 5 μM Zn^{2+} ($n=10$), and 56 ± 9% at 20 mM Zn^{2+} ($n=4$).

Effect of ketamine on ethanol inhibition of NMDA-activated current

We also examined whether ethanol might inhibit the NMDA receptor by interacting with the dissociative anaesthetic binding site. The average percentage inhibition of NMDA-activated current by 50 mM ethanol is given in Table 1 for 0, 2 and 10 μM ketamine. Ketamine did not significantly alter the percentage inhibition by ethanol (ANOVA, $P>0.25$). The inhibition by ketamine in the absence of ethanol was 31 ± 12% at 2 μM ketamine ($n=4$), and 74 ± 4% at 10 μM ketamine ($n=4$).

Discussion

In the study reported here, we investigated the mechanism by which ethanol reduces the amplitude of current activated by NMDA using the whole-cell patch-clamp technique. A number of agents, including ketamine, Mg^{2+} and spermine (at high concentrations) inhibit the NMDA receptor-ion channel complex in a manner dependent upon membrane potential (Mayer *et al*., 1984; Nowak *et al*., 1984; Honey *et al*., 1985; MacDonald *et al*., 1987; Mayer *et al*., 1988; Rock & Macdonald, 1992). In contrast, in the present studies, ethanol inhibition of the NMDA receptor-ion channel complex was not voltage-dependent, whether measured when membrane potential was rapidly changing or when membrane potential was held constant. As ethanol is not charged at physiological pH,

Table 1 Effect of Mg^{2+}, Zn^{2+}, and ketamine on ethanol inhibition of NMDA-activated current

Modulator (μM)	*n*	*Percentage inhibition by 50 mM ethanol*
Mg^{2+}		
0	22	32 ± 3
10	6	34 ± 2
100	8	44 ± 5
500	6	27 ± 9
Zn^{2+}		
0	6	46 ± 7
5	4	36 ± 3
20	4	48 ± 11
Ketamine		
0	6	37 ± 6
2	4	45 ± 7
10	3	36 ± 9

NMDA concentration was 25 μM; results are means$\pm$ s.e.mean.

any voltage-dependence that might have been observed would have resulted from an ethanol-induced conformational change in the NMDA receptor-channel that affected its voltage sensitivity (Hille, 1992). Additionally, the observation that ethanol did not alter the reversal potential of NMDA-activated current indicates that ethanol did not significantly change the ion selectivity of the NMDA receptor-ion channel. Thus, the inhibition by ethanol cannot be attributed to selective reduction of permeation of the NMDA receptor-ion channel by a given ionic species such as Na^+ or Ca^{2+}.

Ethanol and the NMDA/glutamate agonist site of the NMDA receptor

The observation that ethanol decreased the E_{max} of the NMDA concentration–response curve without affecting the EC_{50} value indicates that the mechanism of ethanol inhibition is non-competitive with respect to NMDA. This is consistent with reports from a number of other laboratories. NMDA-evoked release of [^{3}H]noradrenaline from cerebral cortical slices (Göthert & Fink, 1989; Gonzales & Woodward, 1990) or of [^{3}H]dopamine from striatal slices (Woodward & Gonzales, 1990), NMDA-stimulated Ca^{2+} influx in cerebellar granule cells (Rabe & Tabakoff, 1990) or dissociated whole brain cells (Dildy-Mayfield & Leslie, 1991), and NMDA-activated current in *Xenopus* oocytes injected with rat hippocampal mRNA (Dildy-Mayfield & Harris, 1992) have all been reported to be inhibited by ethanol in a non-competitive manner. In addition, in radiolabelled ligand binding experiments in mouse cortical or hippocampal membranes, ethanol did not alter the binding affinity of [^{3}H]L-glutamate or of the NMDA competitive antagonist [^{3}H]CGS 19755 (Snell *et al*., 1993). In one study ethanol was reported to inhibit NMDA-stimulated nitric oxide formation in the presence of Mg^{2+} in an apparently competitive manner (Chandler *et al*., 1994); however, inhibition of the NMDA response by Mg^{2+}, which inhibits NMDA-gated ion channels via open-channel block (Mayer *et al*., 1984; Nowak *et al*., 1984), also appeared to be competitive in this study.

Ethanol and the glycine coagonist site of the NMDA receptor

In contrast to the general agreement that ethanol does not interact with the NMDA/glutamate agonist site of the NMDA receptor, the role of the glycine coagonist site in mediating the effect of ethanol is controversial. In the present study, ethanol decreased the E_{max} but did not alter the EC_{50} value of the concentration–response curve for glycine, which is consistent with a non-competitive mechanism of inhibition with respect to glycine. The results of the present study using mouse hippocampal neurones are in agreement with results of both electrophysiological (Peoples & Weight, 1992; Chu *et al*., 1995; Mirshahi & Woodward, 1995) and biochemical (Gonzales & Woodward, 1990; Woodward, 1994a; Cebers *et al*., 1996) experiments using rat neuronal preparations or rat brain NMDA receptor subunits expressed in *Xenopus* oocytes, in which ethanol inhibited NMDA receptor-mediated responses in a manner that was non-competitive with respect to glycine. Our observations are also consistent with results of radiolabelled ligand binding experiments in mouse cortex and hippocampus demonstrating that ethanol does not interact with the glycine site of the NMDA receptor (Snell *et al*., 1993). In contrast, in several other studies the inhibitory effect of ethanol has been reported to be reversed by high concentrations of glycine (Hoffman *et al*., 1989; Rabe & Tabakoff, 1990; Woodward & Gonzales, 1990; Dildy-Mayfield & Leslie, 1991; Snell *et al*., 1994). Any of several reasons may account for the disparity between the results of these latter studies and those obtained in the present study. In the present study, NMDA receptor-activated ion current was measured directly, whereas in most previous studies in which glycine reversed the effect of ethanol, the activity of the NMDA receptor was assessed indirectly by measuring changes in cytosolic Ca^{2+} concentration or the release of neurotransmitters. In addition, in those previous studies, potential indirect effects of glycine on other sites were not adequately excluded. Thus, any influence of glycine on such processes might obscure effects mediated by the NMDA receptor.

Because changes in subunit composition of NMDA receptors can result in changes in their modulation by ethanol (Koltchine *et al*., 1993; Kuner *et al*., 1993; Masood *et al*., 1994) and glycine (Kutsuwada *et al*., 1992; Ishii *et al*., 1993; Wafford *et al*., 1993), an alternative explanation for the disparate effects of glycine on ethanol inhibition of NMDA receptors among different studies is that the interaction of glycine and ethanol may differ depending upon the subunit composition of the NMDA receptors under investigation. Chu *et al*. (1995) and Buller *et al*. (1995) have attempted to answer this question by expressing different combinations of recombinant NMDA receptor subunits in *Xenopus* oocytes. Chu *et al*. reported that ethanol inhibition of NR1b/NR2A, NR1b/NR2B and NR1b/NR2C subunit combinations was not altered by 7-chlorokynurenic acid, a competitive antagonist of the glycine site on the NMDA receptor. Buller *et al*. (1995) presented evidence that ethanol inhibition of NMDA receptors formed from NR1/NR2B amd NR1/NR2D subunits is primarily non-competitive with glycine, as ethanol did not alter the glycine EC_{50} in these receptors (although the percentage inhibition by ethanol was higher in low glycine for the NR1/NR2D combination). These investigators also reported, however, that there is a competitive component of inhibition for receptors containing the NR2A or NR2C subunits, as ethanol increased the EC_{50} for glycine in those receptors. The discrepancy between the results of Chu *et al*. (1995) and Buller *et al*. (1995) may have resulted from their use of different splice variants of the NR1 subunit, which have been shown to be differentially sensitive to ethanol (Koltchine *et al*., 1993). As NMDA receptor subunits are differentially distributed throughout the brain (Kutsuwada *et al*., 1992; Meguro *et al*., 1992; Monyer *et al*., 1992), neurones from different brain areas may thus express NMDA receptor subunit combinations that differ in their sensitivity to modulation by ethanol and glycine.

Ethanol and the Mg^{2+} site of the NMDA receptor

Studies assessing the effects of ethanol on NMDA receptor-mediated depolarisation in hippocampal slices and on NMDA-stimulated nitric oxide formation in cultured cortical neurones reported that Mg^{2+} increased the potency of ethanol (Martin *et al*., 1991; Morrisett *et al*., 1991; Chandler *et al*., 1994). In contrast, ethanol inhibition of NMDA-activated current in the present study was not affected by changes in Mg^{2+} concentration. The reason for the difference in the effect of Mg^{2+} in the earlier studies and the present experiments is unclear, but this difference may arise from the different techniques used. For example, because NMDA receptor-channel activation was not measured directly in the previous studies, Mg^{2+} may have had effects at sites other than the NMDA receptor, and any interaction between Mg^{2+} and ethanol in influencing these sites could have obscured effects mediated by the NMDA receptor. Such actions of Mg^{2+} at additional sites could account for the increases in the slopes of the NMDA concentration–response curves in the presence of Mg^{2+} observed in those previous studies (Martin *et al*., 1991; Chandler *et al*., 1994). In addition, although it is well established that Mg^{2+} inhibits the NMDA receptor-ion channel via an open-channel block mechanism (Mayer *et al*., 1984; Nowak *et al*., 1984), in one of these studies, Mg^{2+} appeared to inhibit NMDA-stimulated nitric oxide formation in a competitive manner (Chandler *et al*., 1994). The conclusion of the present study that ethanol and Mg^{2+} act at different sites on the NMDA receptor-channel is supported by a number of biochemical experiments in which ethanol did not alter the potency of Mg^{2+} (Gonzales & Woodward, 1990; Rabe & Tabakoff, 1990; Woodward & Gonzales, 1990; Bhave

et al., 1996), by electrophysiological experiments in which Mg^{2+} did not affect ethanol inhibition of the NMDA receptor-ion channel (Chu *et al*., 1995), and by single-channel experiments in which ethanol, unlike Mg^{2+}, did not appear to act via open-channel block (Wright *et al*., 1996).

Ethanol and the oxidation-reduction modulatory site of the NMDA receptor

Ethanol has been previously reported to inhibit NMDA receptor-mediated neurotransmitter release from rat brain slices in a manner that is dependent upon the oxidation state of the receptor (Woodward, 1994a). In contrast, neither the oxidizing agent DTNB nor the reducing agent DTT had any effect on ethanol inhibition of NMDA-activated current in the present study. This difference between the results obtained in the previous study and those of the present study could arise from differences in the NMDA receptor subtypes. Although both studies were performed using neurones or slices from the hippocampus, the NMDA receptors regulating norepinephrine release appear to be located presynaptically, and have been reported to differ in subunit composition from the postsynaptic receptor-channels that mediate NMDA-activated current (Wang & Thukral, 1996).

Ethanol and other modulatory sites of the NMDA receptor

In the present experiments the amplitude of NMDA-activated current was enhanced by spermine and increased pH, and was inhibited by Zn^{2+} and ketamine. The absence of any effect of these agents on the inhibition by ethanol of the NMDA receptor is consistent with the physicochemical differences between these molecules and the ethanol molecule. For example, as ethanol is uncharged at physiological pH, it would not be expected to act upon the same site as a cation such as H^+ or Zn^{2+}. In addition, the characteristics of ethanol inhibition of the NMDA receptor-channel differ in most instances from those of other modulators. For example, ethanol does not appear to inhibit NMDA receptor-channels via an open-channel block mechanism (Wright *et al*., 1996), in contrast to dissociative anaesthetics such as ketamine (Honey *et al*., 1985; MacDonald *et al*., 1987). The findings of the present study are also in agreement with previous studies on other preparations in which the effect of ethanol was not altered in the presence of the polyamine spermine (Rabe & Tabakoff, 1990) or Zn^{2+} (Chu *et al*., 1995).

Site of action of ethanol on the NMDA receptor

A specific site of action of ethanol on the NMDA receptor-ion channel complex remains to be elucidated. However, the findings of the present study, taken together with previous reports that the potency of alcohols for inhibiting NMDA receptor-mediated events is related to hydrophobicity (Lovinger *et al*., 1989; Fink & Göthert, 1990; Lovinger *et al*., 1990a; Gonzales *et al*., 1991), are consistent with an action of ethanol at a hydrophobic site. Although alcohols have been proposed to produce their actions via non-specific perturbation of plasma membrane lipids, evidence from this (Peoples & Weight, 1994; 1995) and other (Dildy-Mayfield *et al*., 1996) laboratories supports the idea that alcohols may act directly on the NMDA receptor-channel protein. For example, inhibition of NMDA-activated current by alcohols exhibits a 'cutoff' effect, which is not explained by a disordering action on the membrane lipid, as lipid disordering continues to increase beyond the cutoff point (Peoples & Weight, 1995). In addition, for a series of alcohols, the relation between hydrophobicity and potency for modulating receptor function differs markedly for NMDA and $GABA_A$ receptors (Peoples & Weight, 1994). Further investigation will be required to elucidate the nature of the site of ethanol action on the NMDA receptor.

We thank Dr Jerry M. Wright for comments on the manuscript and Stephen McCort and Anna-Maria Vasquez for technical assistance.

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(Received March 24, 1997
Accepted August 12, 1997)