Ethanol Inhibits AMPA Receptor Function in CNS Neurons by Stabilizing Desensitization

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Abbreviations: AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, CSF = Cerebrospinal fluid, DMEM = Dulbecco’s modified eagle’s medium, NMDA = N-methyl-D-aspartate

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Abstract

Ethanol actions on AMPA-type glutamate receptors were studied using voltage clamp recordings from mouse cortical and hippocampal neurons. During whole-cell recordings ethanol (EtOH) inhibited AMPA receptor-mediated currents in a dose-dependent manner at concentrations from 10 to 500 mM. The steady-state component of AMPA-activated current was more sensitive to EtOH than the peak component. To examine the effect of EtOH on a well-resolved peak current component, patches were excised from cultured cortical neurons, to which AMPA and EtOH were applied using a piezoelectric solution application system. Under this condition the peak current was not inhibited significantly by EtOH. To further study possible mechanisms of EtOH inhibition, kainate and AMPA were used to evoke currents in the absence and presence of cyclothiazide. Ethanol inhibition was stronger when receptors were activated by low than high kainate concentrations. Cyclothiazide reduced inhibition by EtOH regardless of the agonist used to activate the receptor. Finally, EtOH inhibition was reduced in a point mutated (L497Y) GluRAi receptor that lacks desensitization. These findings suggest that EtOH inhibits AMPA receptors by stabilizing the desensitized state. Our results can explain some of the variation observed in EtOH inhibition in previous studies, and support the idea that physiologically relevant concentrations of EtOH can have a strong effect on AMPA receptor function.
Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS). It produces its physiological actions by activating several metabotropic and ionotropic receptors (see Lovinger, 1997 for review). The different ionotropic glutamate receptors are divided into three subtypes based on their primary amino acid sequence, and the agonists that best activate them. These three classes of receptor have been named the N-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtypes. Glutamate activation of AMPA receptors (AMPARs) is thought to mediate most fast synaptic excitatory neurotransmission in the brain, while transmission via kainate receptors contributes only a minor component.

Ethanol (EtOH) has been shown to act on several targets in CNS, and the glutamate receptors are among these sites of action. Ethanol decreases the function of all three classes of ionotropic receptors at concentrations in the physiologically relevant range (Lovinger et al., 1989; Weiner et al., 1999). Ethanol is also known to inhibit glutamatergic synaptic transmission (Lovinger et al., 1990). While the majority of studies have focused on potent inhibition of NMDA receptors by EtOH, the effect on AMPARs is not as well studied. This is in part due to the fact that EtOH inhibition of AMPA receptors is rather weak in many neuronal preparations (Lovinger et al., 1989; Weiner et al., 1999) and AMPAR-mediated synaptic transmission is not greatly inhibited by EtOH at hippocampal synapses (Lovinger et al. 1990, Weiner et al., 1999). However, reasonably potent inhibition of AMPAR function by EtOH has been observed in heterologous expression systems and in some neuronal preparations when receptor function is studied using direct agonist application to isolated cells or oocytes (Dildy-Mayfield and Harris, 1992; Dildy-Mayfield and Harris, 1995; Lovinger, 1993; Wirkner et al., 2000). These studies demonstrated EtOH inhibition of AMPARs at concentrations ranging from those below the legal intoxication limit (10 mM or ~50 mg/dL) to near lethal concentrations (100 mM or ~500 mg/dL). Wirkner et al. (2000) found that EtOH inhibition of NMDA and AMPA receptors is likely caused by noncompetitive inhibition distinct from open channel block (see also Peoples et al., 1997). In oocytes, EtOH inhibition of AMPARs appears to vary with agonist concentration, with inhibition being greater at low agonist concentrations (Dildy-Mayfield and Harris, 1992). These results
suggest that AMPA receptors might possess considerable EtOH sensitivity under certain conditions or in certain preparations, but little is known about the interactions with receptor-channel function.

AMPA receptors undergo profound desensitization during agonist exposures lasting milliseconds. With the exception of kainate, all of the widely used AMPAR agonists evoke strong desensitization of the receptor (Trussell et al., 1988; Tang et al., 1989). Kainate produces desensitization that is extremely rapid and nearly undetectable, because it is thought to be rapidly reversible, and hence is weak in comparison to the desensitization produced by other agonists (Patneau et al., 1993). The time constants of desensitization in response to AMPA or glutamate measured in outside-out membrane patches have been shown to range from 1 to 16 ms (Tang et al., 1989; Trussell and Fischbach, 1989; Hestrin, 1992; Barbour et al., 1994). It is thought that desensitization does not contribute to AMPAR-mediated transmission at intact synapses (Colquhoun et al., 1992; Hestrin, 1992; Diamond and Jahr, 1995), because of the rapid clearance of the transmitter from the synaptic cleft (Clements et al., 1992). However, this process may come into play in cases of unusually prolonged synaptic transmission, as observed in experiments in various CNS neurons, where the excitatory postsynaptic current (EPSC) decay reflects the rate of desensitization rather than deactivation (Trussell et al., 1993; Barbour et al., 1994; Otis et al., 1996; Maguire, 1999).

The fast time course of desensitization necessitates that rapid drug application should be used to properly resolve the peak current when AMPARs are activated by most agonists. Past studies examining EtOH inhibition of AMPAR function have not taken this factor into account, and there has been no attempt to determine if EtOH alters desensitization or if inhibition varies when receptors are activated under conditions that produce different degrees of desensitization. In the present study we examined the effect of EtOH on AMPARs in acutely isolated as well as cultured CNS neurons. Our aim was to examine the effect of EtOH on different components of AMPAR-mediated current evoked by different agonists. Our findings suggest that EtOH inhibits AMPAR function with a pharmacologically relevant potency, and that inhibition is mainly due to
stabilization of receptor desensitization. Some of the results described in this manuscript have appeared in abstract form (Möykynen et al., 2001).
Methods

Isolation of hippocampal cells

C57Bl/J6 mice (10-20 days old) were decapitated and the whole brains were moved to ice-cold high-sucrose solution (containing in mM: sucrose, 194; NaCl, 30; KCl, 4.5; MgCl₂, 1; NaHCO₃, 26; NaH₂PO₄, 1.2; glucose 10, bubbled with 95 % O₂/ 5 % CO₂ to achieve pH 7.4). Coronal brain slices (400 µm thickness) were cut using a manual vibroslice (Camden Instruments Ltd, Leicester, U.K.). Slices were kept at room temperature in artificial CSF (containing in mM: NaCl, 124; KCl, 4.5; MgCl₂, 1; NaHCO₃, 26; NaH₂PO₄, 1.2; glucose, 10; CaCl₂, 2, bubbled continuously with 95 % O₂/ 5 % CO₂). Hippocampal neurons were isolated by incubating slices in pronase (Calbiochem, San Diego, CA, USA, 0.4-0.6 mg/ml) in aCSF at 37°C for 20 – 30 min. Slices were transferred to trituration buffer (containing in mM: NaCl, 20; N-methyl-D-glucamine, 130; KCl, 2.5; MgCl₂, 1; HEPES, 10; glucose, 10, osmolarity adjusted with sucrose to 340 mOsm, pH adjusted to 7.4 with HCl), and the hippocampus was cut free of the rest of the tissue using a scalpel. Neurons were dissociated by gentle mechanical trituration with fire-polished Pasteur pipettes. Cells were then allowed to settle to the bottom of a 35 mm diameter culture dish, which was then transferred to the stage of an inverted microscope.

Maintenance and Transfection of HEK 293 Cells

Human embryonic kidney (HEK) 293 cells were maintained in culture as previously described (McCool et al. 1996). Cells to be used for transfection were plated onto 35 mm diameter culture dishes in medium containing DMEM with 4.5 g/L glucose, pyridoxine, pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. One to two days after plating cells were transfected via calcium-phosphate precipitation using HEPES-buffered saline solution (HBSS) combined with 2.5 mM CaCl₂ and the appropriate concentration of the cDNAs. Cells were transfected with either wild-type GluRAi (i = flip splice variant, 2-5 micrograms per dish) or the L497Y (numbering refers to full-length protein as in Stern-Bach et al., 1998), mutant GluRAi (2 µg per dish). cDNA encoding Enhanced Green Fluorescent Protein (EGFP, 1-2 µg per dish) was
included in each transfection to mark successfully transfected cells. cDNAs were contained in mammalian expression vectors containing a CMV promoter. Cells were exposed to cDNA for 1 day, and then washed with standard feeding medium. Cells were then examined electrophysiologically beginning 16-24 hours after washing. EGFP positive cells were identified by epifluorescence.

Electrophysiological recordings

During experiments the cells were continuously superfused with recording solution (containing in mM: NaCl, 150; KCl, 2.5; CaCl2, 2.5; MgCl2, 1; HEPES, 10; glucose, 10, pH adjusted to 7.4 with NaOH, osmolarity to 340 mOsm with sucrose). Experiments were carried out at room temperature. Whole-cell and outside-out patch-clamp recording techniques were used (Hamill et al., 1981). Patch pipettes were pulled from 1.5 mm o.d. glass capillary tubing (World Precision Instruments Inc, Sarasota, FL, USA) using a micropipette puller (Sutter Instrument Co, Novato, CA, USA; Model P-87). Pipette resistance was 2-4 MΩ, when filled with an internal solution containing (in mM): N-methyl-D-glucamine, 100; CH3SO3H, 100; CsF, 40; MgCl2, 10; HEPES, 10; EGTA, 5, pH adjusted with CsOH to 7.4, osmolarity 290-300 mOsm. Drugs and EtOH were diluted in recording solution and applied to whole cells with a multi-barrel fast solution application system (Warner Instrument Corp, Hamden, CT, USA). In the majority of experiments, neurons were lifted clear of the bottom of the culture dish to facilitate solution exchange. Ethanol and agonist were applied simultaneously in most experiments. Pre-application of EtOH did not substantially alter experimental outcomes (see results below). To measure the solution exchange times in this system we applied 1 mM kainate to the cells to activate the AMPAR’s. The solution was then changed to a solution lacking Na⁺, which almost completely eliminated the kainate-evoked ion current. The exchange time for cells sitting in the bottom of the dish was 150 ± 60 ms and for lifted cells 22 ± 5 ms. Drug applications varied in duration from 100 ms to 8 s. Under the conditions used, the transient peak current component was observed only when the solution delivery pipettes were placed directly above the cells with the solution stream flowing directly onto the cell.
An Axopatch 1-D or Multiclamp amplifier and pClamp6.0 or pClamp8.0 software (Axon Instrument, Inc., Union City, CA, USA) were used to acquire and analyze current recordings. During experiments neurons were voltage clamped at –60 mV, unless otherwise mentioned. Currents were filtered with a 5 kHz low pass filter and digitized at up to 100 kHz.

To better resolve EtOH effects on pre-desensitized peak currents we used a piezoelectric application system (model PZS-200, Burleigh Instruments, Inc., Victor, NY) to apply drugs to excised membrane patches with exchange times, measured for open tip potential change, being 4 ± 2 ms. Patches were pulled from cultured cortical neurons, which were made from mice at postnatal days 1 and 2 using methods described in Strack et al. (1999). The experiments were carried out after 7-14 days in culture. The drug application pipette was made from a theta tube glass capillary (Warner Instrument Corporation, Hamden, CT, USA). The outer diameter was cut to about 150 µm. The recording electrode was placed at an angle of ~90 degrees with respect to the drug application pipette. Current evoked by 100 µM AMPA in the absence and presence of 100 mM EtOH was examined using drug application durations of 1 and 700 ms.

In all experiments, each drug application was repeated at least twice and averaged. Control applications, agonist without EtOH, were measured at least twice between every pair of applications of agonist with EtOH. The effect of EtOH was calculated as percent inhibition relative to the averaged control values before and after EtOH application. EtOH concentration-response curves were well fit using a single binding-site isotherm of the form:

$$Y = \frac{B_{max} \cdot X}{(K_d + X)}$$

AMPA concentration-response curves were fit with a standard 4-parameter logistic equation of the form:

$$Y = E_{min} + (E_{max} - E_{min}) \left[ 1 + \left( \log EC_{50} - X \right)^n \right]$$
Where Emin is the minimal response, Emax is the maximal response, EC_{50} is the concentration giving a half-maximal response, X is log concentration, and n is the hill slope factor. No parameters were constrained during curve fitting.

Results are given as mean ± SEM. Statistical differences among groups were determined using ANOVA with post-hoc comparisons using the Bonferroni test, and in some cases with a one sample t-test. The criterion for statistical significance was p<0.05.

All drugs were purchased from commercial sources. S-AMPA, kainate, SYM 2081 and cyclothiazide were purchased from Tocris Cookson Inc (Ballwin, MO, USA). Ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA). Concanavalin A was from Sigma Chemical Co. (St. Louis, MO).
Results

Characterization of AMPA-evoked current in isolated hippocampal pyramidal neurons

Application of AMPA for 8 s to isolated hippocampal pyramidal neurons at a holding potential of –60 mV evoked inward currents of stable amplitude with characteristic fast peaks followed by a steady-state phase (Fig. 1A). Increasing concentrations of AMPA (1 – 1000 µM) were applied to determine the concentration-response relationship for this agonist (Fig. 1B). Concentration-response curves were fit with the logistic equation as shown in Fig. 1B and estimated EC40 (10 µM) and EC80 (100µM) concentrations of AMPA from steady-state responses were used in later experiments.

Ethanol inhibition of AMPA-mediated current

Ethanol inhibited AMPA-evoked current in a concentration-dependent manner in all isolated neurons measured. Figure 2A and B show that EtOH produced prominent inhibition of the steady-state component of current activated by 100 µM AMPA as well as the peak and steady-state components of current activated by 10 µM AMPA. Inhibition by EtOH was reproducible with multiple drug applications, and reductions in current amplitude were fully reversed upon removal of EtOH from the cell (Fig. 2). Figure 2C also illustrates the stability of AMPA-evoked currents during prolonged recording from isolated hippocampal neurons.

Previous studies had suggested that EtOH inhibition of AMPARs in heterologous systems differed at different agonist concentrations (Dildy-Mayfield and Harris, 1992). We thus examined EtOH inhibition when the AMPAR was activated by either 10 (EC40), or 100 µM AMPA (EC80) in isolated hippocampal neurons (Fig. 3). EtOH inhibition of the agonist-activated peak current was significantly greater when receptors were activated by 10 µM as compared to 100 µM AMPA (Fig. 3A). In contrast, no agonist concentration-dependent difference in inhibition of steady-state current was detected (Fig. 3B).
The dependence of EtOH inhibition on agonist concentration might reflect an interaction with the binding of agonist, but could also result from an interaction with channel gating properties that are dependent on agonist concentration. To begin to assess this latter possibility, we examined EtOH effects on several components of the whole-cell current, including the rapid-onset, transient peak of AMPA-evoked current, the subsequent exponential decay, and the steady-state component observed during continued agonist applications lasting 8 s, because these aspects of whole-cell current likely result from differential contributions of various channel state transitions such as desensitization/resensitization of the receptor-channel. By comparing EtOH inhibition of these different current components in isolated hippocampal neurons we hoped to gain an initial idea about what channel properties are altered by EtOH. In order to see distinct peak and steady-state responses a reasonably high agonist concentration, 100 µM AMPA, was used. Both the peak and steady-state current components were inhibited by EtOH (see example responses in Fig. 2B). However, EtOH produced significantly greater inhibition of the steady-state than the peak response at all concentrations tested. For example, the inhibition by 100 mM ethanol of steady-state current was 35 ± 2 % (mean ± SEM, n = 13) and only 15 ± 1 % for the peak response (Fig. 3C). Application of EtOH for at least 1 minute prior to combined AMPA + EtOH application did not alter the relative inhibition of peak and steady-state current components. In a group of 7 neurons 100 mM EtOH produced 19+/-2% inhibition of peak current with EtOH pre-application or 14+/-3% without preapplication, while inhibition of steady-state current averaged 30+/-3% with and 33+/-2% without EtOH preapplication. Steady-state/peak ratios for 100 µM AMPA with and without EtOH were also calculated for these recordings. The ratio decreased with increasing alcohol concentration (Fig. 3D).

The time course of the exponential decay of 100 µM AMPA-activated currents was determined using non-linear curve fitting in neurons lifted from the bottom of the culture dish (to enhance solution exchange rate). Decay was well fit with a single exponential function both in the absence and presence of EtOH. The time constant (τ) for decay of current during agonist exposure was similar in the presence and absence of EtOH (for 50 mM EtOH experiments, baseline = 42 +/- 7 ms, EtOH = 42 +/- 7 ms; for
Ethanol does not alter AMPA-induced peak current in excised membrane patches

Membrane patches were excised from cultured cortical neurons to which drugs were applied using the piezoelectric applicator. The fast application system allowed drugs to be applied to patches with an exchange time of 4+/2 ms, faster than the time constant of desensitization. Thus, we could more accurately resolve the peak of AMPA-induced current using this system. Ethanol, even at the 100 mM concentration, did not affect the peak amplitude of current evoked by 100 µM AMPA [current in the presence of 100 mM EtOH averaged 99 ± 3 % of the amplitude of control currents (Fig. 4A)]. The average time constant for current decay in the presence of agonist was 11.1 ± 1.7 ms, which is longer than the solution exchange time in our system (Fig. 4B), and is lower than that observed in the whole-cell mode. The decay time constant in the presence of agonist and 100 mM EtOH averaged 10.8 ± 1.8 ms, which was not significantly different from agonist alone (P>0.05, paired t-test, n=5 patches). The steady-state component of current in excised membrane patches was too small to be resolved, as shown in figure 4A, and thus we could not estimate EtOH inhibition of this current component under this condition. EtOH inhibition of the steady-state current in whole-cell recordings from cultured cortical neurons was confirmed to be similar to that observed in isolated hippocampal neurons (~40%) using the stepper motor driven solution applicator (data not shown).

We also examined recovery from desensitization of 100 µM AMPA-induced current in the absence and presence of 100 mM EtOH in isolated hippocampal neurons. This was accomplished using a two-pulse protocol in which AMPA was applied for 100 ms followed by a wash period of 200-1000 ms, and subsequent re-application of AMPA (Fig. 5). When the effect of EtOH was examined, EtOH was applied throughout the duration of the recording protocol regardless of whether or not agonist was present. The peak amplitude of AMPA-induced current recovered with a time constant of 189 ms (95% confidence interval 175-204 ms, n=9 cells) and this value increased to 233 ms.
(95% confidence interval 217-250 ms, n=9 cells) in the presence of EtOH (Fig. 5B). Thus, EtOH produced a modest slowing of the rate of recovery from desensitization.

**Ethanol Inhibition Varies with Kainate Concentration**

Kainate activates AMPA receptors in a manner that produces extremely rapid desensitization and resensitization, resulting in steady-state desensitization that is less complete than that produced by AMPA. Application of kainate (30 µM - 1 mM) to isolated hippocampal neurons produced current that showed no decay during agonist applications of up to 8 sec. When receptors were activated by a maximally-effective concentration of kainate (1 mM), relatively little inhibition was observed (only 23 +/- 2 % in the presence of 100 mM EtOH). This was considerably less than the inhibition produced by 100 mM EtOH on the steady-state component of current produced by 100 µM AMPA (see figure 3 above). This result is not surprising given that overall receptor desensitization is less in the presence of the high kainate concentration in comparison to the high AMPA concentration. However, it is possible that EtOH might produce greater inhibition of responses to lower kainate concentrations where receptor occupancy is lower and more receptors desensitize from a single agonist bound state (Patneau and Mayer 1991). Indeed, it was previously reported that EtOH inhibition of kainate-evoked currents from *Xenopus* oocytes expressing rat hippocampal and cortical mRNA decreased with increasing agonist concentration (Dildy-Mayfield and Harris, 1992). To determine if inhibition was sensitive to kainate concentration in isolated hippocampal neurons, we examined EtOH effects in the presence of 30 and 300 µM kainate, and compared the inhibition to that observed in the presence of 1 mM kainate. We observed a marked EtOH inhibition of low (30 µM) kainate-evoked currents (Fig. 6A). Figure 6B shows that inhibition produced by EtOH was dependent on both agonist and EtOH concentrations and was greater at low than high kainate concentrations. When we performed within-cell comparisons, we observed that 100 mM EtOH produced 44 ± 4 % inhibition of 30 µM kainate-evoked currents, whereas the inhibition was only 23 ± 13 % for currents activated by 300 µM kainate (P<0.05, n = 6).
Kainate is also an agonist for kainate-type glutamate receptors, and therefore it was necessary to determine if functional kainate receptors contributed to the current we observed. The currents activated by kainate in our isolated neurons did not exhibit the rapid decay during agonist application that is characteristic of kainate activation of kainate-preferring ionotropic glutamate receptors (Ozawa et al., 1998). Thus it is unlikely that kainate receptors are involved in generation of the currents we measured.

To be sure that kainate-activated currents could not be evoked in the isolated neurons, a selective kainate receptor agonist, SYM 2081 (Donevan et al., 1998), was applied to neurons in the presence of Concanavalin A (Con A) to block rapid desensitization. SYM 2081 at a concentration of 2 µM along with 0.3 mg/ml Con A (SYM 2081 and Con A concentrations that produced near-maximal activation of kainate-preferring GluRs, Donevan et al., 1998) evoked currents with amplitudes smaller than 5 pA in the neurons examined (data not shown). Normal responses to 100 µM AMPA and EtOH inhibition of AMPA-induced current were observed in these same neurons. These findings suggest that kainate-activated current is mediated solely through AMPA receptors in the neurons examined.

Cyclothiazide reduces EtOH inhibition of AMPA-activated current

Cyclothiazide stabilizes the open state of AMPA receptors (Kessler et al., 1996), and one effect of this compound is to greatly reduce desensitization. If EtOH inhibits AMPAR function by stabilizing desensitization we would expect this inhibition to be reduced in the presence of cyclothiazide. We thus examined EtOH (50 and 100 mM) effects on current activated by 10 µM AMPA with and without 100 µM cyclothiazide in isolated hippocampal neurons. Cyclothiazide produced a profound enhancement of the peak AMPA-activated current, and eliminated the fast, transient current component (Fig. 7A). Ethanol produced greater inhibition of current activated by AMPA alone in comparison to current evoked by AMPA and cyclothiazide. Inhibition by 100 mM ethanol averaged 39 ± 6 % and 21 ± 6 % in the absence and presence of cyclothiazide, respectively (Fig. 7B). However, current in the presence of cyclothiazide, measured at – 60 mV, had amplitudes in the nA range, and thus were substantially larger in amplitude than those evoked by AMPA alone. It is, therefore, possible that reduced inhibition by
EtOH might reflect problems with voltage- or space-clamp. To determine if clamp-control problems contribute to the measured EtOH inhibition, we examined AMPA-activated current in the absence and presence of cyclothiazide at lower holding potentials so that current was reduced by reducing the driving force. Lowering the holding potential decreased the amplitudes of responses to a range of a few hundred pA. No differences in ethanol inhibition were observed when comparing the results at -60 mV and -20 and -10 mV. EtOH (100 mM) inhibited currents 19 ± 5 % at -20 to -10 mV and 19 ± 4 % at -60 mV (n = 5). This finding indicates that EtOH inhibition is not dependent on current amplitude, and that clamp-control problems are unlikely to account for the differential inhibition of current in the absence and presence of cyclothiazide. These results support the idea that there is a difference in the EtOH sensitivity of AMPA receptors that undergo desensitization relative to those that do not.

Cyclothiazide also reduced EtOH inhibition of kainate-activated current (Fig. 7C). In the presence of 100 µM cyclothiazide the inhibition of 30 µM kainate-activated current by 100 mM EtOH was only 21 ± 2 %, whereas without cyclothiazide it was 44 ± 4 %, suggesting that disruption of desensitization alters EtOH inhibition when kainate is used as agonist.

**Ethanol Inhibition in WT and L497Y Mutant GluRAi**

To further explore the relationship between desensitization of AMPA-type glutamate receptors and EtOH inhibition, we examined alcohol effects in wild-type GluRAi receptors in comparison to a mutant receptor (L497Y) that lacks desensitization (Stern-Bach et al. 1998). The receptors were expressed transiently in HEK 293 cells after transfection with the appropriate cDNA along with EGFP as a marker. Transfected cells were examined with whole-cell patch-clamp recording. Agonist and alcohol application was initiated after lifting cells from the bottom of the culture dish, as described above. Application of 100 µM AMPA produced currents that exhibited rapid desensitization. Application of EtOH produced dose-dependent inhibition of current activated by 100 µM AMPA in HEK 293 cells expressing wild-type GluRAi receptors (Fig. 8). Inhibition of the steady-state current component was greater than inhibition of peak current, as
observed in neurons. Inhibition of the steady-state current component was comparable in magnitude to that observed in neurons (% inhibition in wild-type GluRAi = 46 ± 2 %). The current activated by 10 µM AMPA was insufficiently large in amplitude in these cells to allow measurement of inhibition by EtOH.

As previously reported, application of 10 or 100 µM AMPA to cells expressing L497Y receptors evoked relatively large amplitude currents that showed little or no evidence of desensitization. Ethanol inhibition was greatly reduced in the L497Y mutant receptor (Fig. 8E). Inhibition averaged only 22 ± 2 % at 100 mM EtOH in the presence of 10 µM AMPA, and inhibition by 100 mM EtOH averaged 19 ± 1 % when receptors were activated by 100 µM AMPA.
Discussion

The results reported at present provide additional evidence that EtOH inhibits AMPAR function. Indeed, inhibition of steady-state current activated by AMPA, and current activated by low concentrations of kainate is comparable in magnitude to that observed at NMDA receptors in several preparations (Dildy-Mayfield and Harris, 1992; Nie et al., 1994; Martin et al., 1995; Wirkner et al., 2000). Our observations may also help to explain some aspects of EtOH inhibition of AMPARs heterologously expressed in Xenopus oocytes (Dildy-Mayfield and Harris, 1992). The steady-state component of current is the only component that is observed in Xenopus oocytes, given the slow superfusion of these large cells. Thus, the EtOH inhibition observed in the oocyte preparation almost certainly reflects changes in steady-state current. In previous studies AMPARs were shown to be inhibited by EtOH if agonist was applied directly to cells, whether the cells were isolated or in brain slices (Dildy-Mayfield and Harris, 1992; Weiner et al., 1999; Wirkner et al., 2000). The drug application systems used for these studies had relatively slow exchange times, and applications lasted for seconds. Thus, the measured currents mainly reflect conditions under which desensitization has been initiated.

The observation that EtOH inhibits the steady-state component of AMPA-activated current to a greater extent than the peak current is consistent with the idea that EtOH promotes or stabilizes receptor desensitization. Previous studies have suggested that the steady-state current reflects openings from the desensitized state in preference to non-desensitized states. Indeed, it has been estimated that steady-state current in neurons reflects openings from 90% of desensitized and 10% of non-desensitized channels (Vyklicky et al., 1991). The desensitized receptor state is energetically the most favorable state of the agonist-bound receptor. Inhibition of peak current was minimal in outside-out membrane patches. These experiments are particularly important for interpretation of this finding because the solution exchange in this preparation is sufficiently fast to allow us to measure a large number of pre-desensitized receptors.
We observed a difference in EtOH inhibition between the peak and steady-state currents when 100 µM, but not when 10 µM AMPA, was used as agonist. When the low agonist concentration was used with a relatively slow agonist application system, a distinctive peak response could not be consistently produced. Peak response evoked by a low concentration of agonist is thus most likely composed of the mixture of openings from non-desensitized and desensitized states. Therefore it is not surprising that we did not see any difference in EtOH inhibition between peak and steady-state response at the 10 µM AMPA concentration.

An interaction between EtOH and desensitization is also supported by our pharmacological experiments. Application of cyclothiazide greatly reduces desensitization, and this compound also significantly reduced EtOH inhibition of the receptor. While it is possible that cyclothiazide counteracts EtOH effects by preventing other avenues of channel closing (e.g., deactivation/unbinding of agonist), our findings with cyclothiazide, together with the lack of effect of EtOH on initial current amplitude, argue for an interaction with desensitization rather than deactivation.

Evidence supporting an EtOH effect on AMPAR desensitization also comes from our experiments on the L497Y GluRAi mutant that lacks desensitization. Ethanol inhibition is clearly reduced, and nearly abolished at lower EtOH concentrations, in this mutant receptor. This contrasts with the wild-type GluRAi receptor that shows normal levels of desensitization, and inhibition by EtOH that is comparable to that observed in neurons. As observed in neurons, inhibition by EtOH was greater for the steady-state than for the peak current component in the wild-type GluRAi receptor. Inhibition of the non-desensitizing mutant receptor is similar in magnitude to that observed in the neurons in the presence of cyclothiazide (see figs. 7 and 8), supporting the idea that cyclothiazide reduces EtOH inhibition by reducing desensitization. It must be noted that a significant level of inhibition remains in this mutant receptor and in the presence of cyclothiazide. This could be due to EtOH effects that counteract the actions of cyclothiazide and the mutation, allowing some degree of desensitization. Alternatively, EtOH may inhibit receptors via an additional mechanism that does not involve desensitization. Thus, in
addition to interactions with desensitization there may be other mechanisms involved in EtOH inhibition of AMPARs.

Interestingly, a recent preliminary report (Akinshola and Taylor, 2001) indicates EtOH inhibition of AMPARs is diminished by a point mutation in the large extracellular loop S2, within the flip/flop alternatively spliced region where the cyclothiazide binding site is thought to reside (Partin et al.; 1995, Sommer et al. 1990). This finding provides additional support for an interaction of EtOH with AMPAR desensitization. In the recently characterized structure of the S1 and S2 regions of GluR2, this portion of the S2 loop lies in close proximity to the residue equivalent to L497 in GluR1 (Sun et al. 2002). Thus, this region of the receptor may be part of a site for alcohol interaction with the protein. A direct interaction between cyclothiazide and EtOH within this region of the protein is also possible, such that the compounds can affect one another’s actions at the receptor. This possible interaction will be an interesting subject for future studies.

The observation that EtOH inhibits kainate-induced responses in an agonist concentration-dependent manner is also consistent with an effect on desensitization. Kainate is a low-affinity partial agonist that produces rapid, but rapidly reversible, desensitization (Patneau et al., 1993). Thus, EtOH effects on desensitization could certainly underlie inhibition when this agonist activates the receptor. One possible explanation for differential EtOH inhibition at different agonist concentrations is that EtOH stabilizes desensitization occurring in the single-agonist-bound state, thus increasing the proportional time the receptor spends in the low conductance-state (at low agonist concentrations) relative to the situation in which desensitization occurs mainly from the high conductance (multiple-liganded) state (at high agonist concentrations). The observation that cyclothiazide reduces EtOH inhibition when kainate is the agonist is also consistent with stabilization of the desensitized state because cyclothiazide would reduce kainate-induced desensitization.

Our findings do not support an effect of EtOH on agonist affinity. We observed that EtOH inhibition of steady-state AMPA-induced current was similar regardless of the agonist concentration used. This finding suggests that EtOH inhibition is not dependent
on receptor occupancy. Furthermore, because the steady-state current represents the highest affinity state of the receptor, we would expect to observe less, rather than more, inhibition of this component of current in relation to peak current if EtOH were only acting as a competitive antagonist. Our findings are consistent with those of Dildy-Mayfield and Harris (1992) who showed that EtOH did not alter the EC₅₀ for kainate activation of GluRs in *Xenopus* oocytes.

Ethanol may produce its inhibitory action by altering the kinetics of the transition(s) leading to the desensitized state (i.e. desensitization) or slowing the transitions out of the desensitized state (i.e. resensitization). The measurements of current decay during agonist exposure indicate that the rate of desensitization appears unaltered in the presence of EtOH. Thus, our results are most consistent with stabilization of the desensitized state due to slowing of resensitization. The modest slowing of resensitization in the presence of EtOH (Fig. 5) supports this interpretation.

Many studies have shown that AMPA receptor-mediated synaptic responses are only weakly inhibited by EtOH at concentrations up to 100 mM (Lovinger et al., 1990; Weiner et al., 1999). In these same studies it was shown that NMDA and kainate receptor-mediated transmission is inhibited to a much greater extent by EtOH. In the past it was not clear why greater EtOH inhibition of AMPA receptors was observed when receptors were activated by agonist application to isolated cells than was observed at intact synapses. The findings in the present paper suggest a probable explanation for this apparent discrepancy, namely that EtOH inhibition of the peak, pre-steady-state, component of AMPA-activated current is much less pronounced than inhibition of the steady-state current component. At the large majority of synapses, it appears that fast desensitization does not contribute to AMPAR-mediated synaptic responses (Colquhoun et al., 1992; Hestrin, 1992; Diamond and Jahr, 1995), because of the short duration of neurotransmitter presence in the synaptic cleft. Thus, the EtOH inhibition at an intact synapse would most likely reflect weak inhibition of pre-desensitized receptors. Of course, it is possible that the differential EtOH sensitivity of agonist-induced versus synaptic responses mediated by AMPARs might also involve a variety of factors including differences in subunit, splice variant, or editing variant composition,
posttranslational modifications, age-related receptor modifications, and EtOH effects on presynaptic function.

These findings raise the question of whether EtOH inhibition of AMPARs plays any part in the neurophysiological effects of the drug \textit{in vivo}. While we cannot answer this question at present, our findings do suggest that EtOH might impact receptor function during conditions where AMPAR desensitization comes into play, such as during periods of prolonged extracellular glutamate release, as might happen during epileptiform activity (Jarvie et al., 1990) or during induction of long-term potentiation\textsubscript{1} or at those synapses where desensitization appears to play a role in shaping AMPAR-mediated synaptic responses (Barbour et al., 1994; Otis et al., 1996) and (Maguire, 1999). Since AMPAR desensitization has been shown to vary with different subunit compositions (Geiger et al., 1995), receptors from different brain areas and different developmental stages may show differences in EtOH sensitivity, which may explain some of the brain regional variability of EtOH effects on neurophysiology.
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References


Footnotes

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Figure Legends

Figure 1. AMPA receptor currents in acutely isolated Hippocampal neurons.

A) Traces showing current activated by increasing concentrations (1, 100 and 1000 µM) of AMPA. Notice the distinctive peak and steady-state current components. All recordings are from the same neuron. Inset shows the current traces on an expanded time scale. B) Concentration-response curves for peak and steady-state currents (n=7 cells at all concentrations). Values are mean ± SEM. Parameters estimated from best-fit curves using the logistic equation: EC$_{50}$ = 110 µM for peak, 28 µM for steady-state, hill coefficient = 1.1 for peak, 0.56 for steady-state.

Figure 2. Reversible EtOH inhibition of AMPA-evoked currents in acutely isolated Hippocampal neurons. A) Current activated by 10 µM AMPA before, during and after application of the indicated concentrations of EtOH. Note that EtOH decreases AMPA-evoked current, and that inhibition is completely reversed upon removal of EtOH from the cell. B) Top: Responses to 100 µM AMPA before during and after EtOH exposure. Bottom: Portions of the traces shown above with an expanded time base to show peak (left) and steady-state (right) current before and during EtOH application. Lines above current traces in A and B indicate duration of agonist alone or agonist plus EtOH application. C) Graph showing the amplitude of peak and steady-state AMPA-activated current as a function of time during the course of an experiment examining repeated applications of different alcohol and AMPA concentrations in a single hippocampal neuron. The data plotted in this graph are all the responses recorded starting from the
first agonist application to this neuron. Note the lack of any “rundown” of current and the reversibility, concentration dependence and repeatability of EtOH inhibition.

**Figure 3. Preferential EtOH inhibition of steady-state AMPA-activated current in isolated Hippocampal neurons.** A) EtOH inhibition of peak current evoked by 10 and 100 µM AMPA. Two-way ANOVA revealed significant differences in inhibition under the 10 and 100 µM AMPA conditions \[F(3,111) = 17.08, P < 0.0001\] and across EtOH concentrations \[F(1,111) = 28.22, P< 0.0001\]. B) EtOH inhibition of steady-state current activated by the different AMPA concentrations. No significant difference was observed for EtOH inhibition at the two different agonist concentrations. Lines in A and B are not best-fit curves. C) EtOH inhibition of peak and steady-state currents activated by 100 µM AMPA \[F (3,84) = 56.54, P < 0.0001\] for the difference between peak and steady-state and \[F (1,84) = 88.27, P < 0.0001\] for EtOH concentrations, 2-way ANOVA-test].

Numbers of neurons examined at each AMPA concentration in B-D: 10 µM AMPA = 6 at 10 mM EtOH, 19 at 25 mM, 26 at 50 mM and 23 at 100 mM; for 100 µM AMPA = 4 at 10 mM, 13 at 25 mM, 17 at 50 mM, 19 at 100 mM, 6 at 200 mM and 6 and 500 mM.

Data are fit with a single binding-site isotherm with best-fit estimated parameters: \(B_{\text{max}} = 116\) and \(K_d = 224\) for \(I_{ss}\), and \(B_{\text{max}} = 138\) and \(K_d = 852\) for \(I_{\text{peak}}\). D) Steady-state / peak (SS/Pk) current ratios in the presence of the indicated were calculated from currents activated by 100 µM AMPA in the absence and presence of EtOH in a group of 6 neurons. In this graph the SS/Pk ratio in the presence of the indicated EtOH concentrations has been normalized to the value in the absence of EtOH for each neuron. One-way ANOVA on the raw SS/Pk ratios indicated a significant effect of EtOH, \(F = \)
(2,5) = 71.62, p<0.0001. Posthoc Bonferroni test revealed significant decrease in SS/Pk ratio in the presence 100, 200 and 500 mM EtOH relative the other two groups (p<0.05). Values in all graphs are mean+-SEM. Line is not best-fit curve.

Figure 4. Lack of EtOH inhibition of peak current activated by fast AMPA application in outside-out patches from cultured cortical neurons. A) Traces show current recorded in excised outside-out membrane patches from cultured cortical neurons activated using 100 µM AMPA application with a piezoelectric system. Lefthand panel shows entire duration of traces, and righthand panel shows same traces with expanded time scale. Ten individual traces were averaged for each condition. EtOH did not significantly inhibit peak current in this experiment. Line above traces indicates duration of pulse used to drive the piezoelectric manipulator. B) The decay phase of current in the presence of EtOH was fit with a single exponential function (gray curve), and this is shown on top of the averaged current traces (black) in the presence and absence of 100 mM EtOH. Current decay is well fit by a single exponential under both conditions, suggesting a single desensitization process in the absence and presence of EtOH. The tau values for decay in these two traces were 8.5 ms in AMPA alone and 9 ms in AMPA + EtOH.

Figure 5. Recovery from desensitization in the absence and presence of EtOH in acutely isolated Hippocampal neurons. A) Current activated by application of 100 µM AMPA delivered using the two pulse protocol in the absence (black traces) and presence (gray traces) of 100 mM EtOH. Current and time scales apply to both sets of
traces. See text for description of two-pulse agonist application protocol. B) Amplitude of peak current activated by 2nd pulse of a two pulse protocol relative to the response to the first pulse (P2/P1) as a function of time following the end of the first agonist application. Data are mean ± SEM ratio values from 9 neurons examined both in the absence and presence of 100 mM EtOH. Lines show the best-fit exponential functions of the recovery curve under each condition.

**Figure 6. Inhibition by EtOH is enhanced at lower kainate concentrations in acutely isolated Hippocampal neurons.** A) (Left) Currents activated by 30 µM kainate in the absence and presence of the indicated concentrations of EtOH. (Right) Currents activated by 1 mM kainate in the absence and presence of EtOH. Lines above traces indicate duration of agonist and alcohol application. B) Average percent inhibition by different concentrations of EtOH when receptors were activated by different kainate concentrations. We observed greater EtOH inhibition when the low kainate concentration was used [Two-way ANOVA test yielded a significant kainate effect F(1,59) = 63.10, P < 0.0001, and significant EtOH effect F(2,59) = 16.33, P < 0.001]. *** P < 0.001 for the difference from 30 µM kainate (Bonferroni test). Values in all graphs are mean ± SEM. Number of neurons examine with 30 µM kainate = 11 at all concentrations, for 300 µM kainate = 6 at all concentrations, and for 1 mM kainate = 7 at 25 mM, 11 at 50 mM and 14 at 100 mM. Lines connecting points are not best-fit curves.

**Figure 7. Cyclothiazide reduces inhibition by EtOH in acutely isolated Hippocampal neurons.** A) Current activated by AMPA + cyclothiazide in the absence
and presence of the indicated EtOH concentrations. Lines above traces indicate the continuous presence of cyclothiazide and the duration of agonist and alcohol application.

B) Mean ± SEM inhibition of peak 10 µM AMPA-induced current in the absence and presence of 100 µM cyclothiazide (data from the same neurons examined under both conditions, n = 7). Two-way ANOVA revealed significant effects of cyclothiazide on EtOH inhibition, [F(1,24) = 10.51, P < 0.005, and EtOH concentration, F(1,24) = 7.81, P = 0.01]. ** P < 0.01 for the difference from the corresponding AMPA alone condition. Number of cells is 7 under all conditions. C) Inhibition of current activated by 30 µM kainate by different EtOH concentrations in the absence and presence of 100 µM cyclothiazide. Note in B and C that inhibition is reduced in the presence of cyclothiazide. Number of cells = 11 at 30 µM kainate and for 1 mM kainate 7 at 25 mM, 11 at 50 mM and 14 at 100 mM EtOH. Two-way ANOVA revealed significant effects of cyclothiazide on EtOH inhibition of kainate-activated current, F(1,32) = 29.89, P < 0.0001, and EtOH concentration, F(1,32) = 5.65, P < 0.05. ** P < 0.01 for the difference from 30 µM kainate (Bonferroni test). Values in all graphs are Mean ± SEM. Lines connecting points are not best-fit curves.

**Figure 8. Ethanol inhibition is reduced in L497Y desensitization-deficient mutant GluRAi receptors.** A) Ion current activated by application of 100 µM AMPA to a HEK 293 cell expressing the wild-type GluRAi receptor in the presence and absence of 100 mM EtOH. B) Current activated by 10 µM AMPA in a HEK 293 cell expressing the L497Y mutant receptor in the presence and absence of 50 and 100 mM EtOH. Note the lack of desensitization in the mutant receptor. C and D) Current traces shown on
expanded time scales to highlight the peak (D) and steady-state (D) wild-type current components activated in the wild-type receptor-mediated current shown in A. Note the marked inhibition of the steady-state component and small inhibition of peak current. E) Graph summarizing the mean ± SEM EtOH inhibition of peak and steady-state current mediated by wild-type receptors at the indicated EtOH concentrations, and inhibition of maximal current activated by 100 µM and 10 µM AMPA in the L497Y mutant receptor. Note the low levels of inhibition relative to inhibition of steady-state current in the wild-type receptor. Two-way ANOVA revealed significant effects of group F(3,58) = 278.5, <0.0001 and EtOH concentration F(2,58) = 334 p<0.0001. Post-hoc analysis with Bonferroni multiple comparison test revealed significant (p<0.05) differences between the 50 and 100 mM EtOH concentrations in each group, significant differences between the 25 and 50 mM EtOH concentrations in the WT SS group, significant differences between the L497Y 100 AMPA and L497Y 10 AMPA at 50 mM EtOH, significant differences between the L497Y 100 AMPA and all other groups at 25 mM EtOH, and significant differences between the WT SS group and all other groups at all EtOH concentrations. Numbers of cells examined for wild-type receptor = 6 at 25 mM EtOH, 5 at 50 mM and 9 at 100 mM EtOH; for mutant receptor = 9 at 25 mM, 9 at 50 mM and 14 at 100 mM.
Moykkynen et al. Figure 2

A

B

C

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Moykkynen et al. Figure 3

A. Peak Current

- 100 µM AMPA
- 10 µM AMPA

B. Steady-State Current

- 100 µM AMPA
- 10 µM AMPA

C. % Inhibition

- SS
- Peak

D. Normalized SS/Pk
Moykkynen et al. Figure 4

A

100 µM AMPA (baseline)

AMPB + 100 mM EtOH

B
Moykkynen et al. Figure 5

A

I (pA)

Control
EtOH

Time (s)

B

P2/P1

Interval Between Applications (ms)
Moykkynen et al. Figure 6

A

100 pA

2.5 s

KA + 100 mM EtOH
KA + 25 mM EtOH
KA + 10 mM EtOH
30 µM KA alone

1 nA

KA + 100 mM EtOH
KA + 50 mM EtOH
1 mM KA alone

B

% inhibition

EtOH (mM)

30 kainate
300 kainate
1000 kainate
A 100 µM cyclothiazide
10 µM AMPA +
0, 50, 100 mM EtOH

B

% inhibition

50 100

0 10 20 30 40 50

EtOH (mM)

KA 30
KA 30 + cyclo

% inhibition

0 10 20 30 40 50

0 25 50 75 100

EtOH (mM)

Moykkynen et al. Figure 7