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Ethanol Inhibition of Synaptically Evoked Kainate Responses in Rat Hippocampal CA3 Pyramidal Neurons

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ABSTRACT

Many studies have demonstrated that intoxicating concentrations of ethanol (10–100 mM) can selectively inhibit the component of glutamatergic synaptic transmission mediated by *N*-methyl-D-aspartate (NMDA) receptors while having little or no effect on excitatory synaptic transmission mediated by non-NMDA receptors [i.e., α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and/or kainate (KA) receptors]. However, until the recent development of highly selective AMPA receptor antagonists, it was not possible to assess the relative contribution of AMPA and KA receptors to non-NMDA receptor-mediated synaptic transmission or to determine whether these glutamate receptor subtypes differed in their sensitivity to ethanol. In the present experiments, we used the highly selective AMPA receptor antagonist LY 303070 to pharmacologically isolate KA receptor-mediated excitatory postsynaptic currents (EPSCs) in rat hippocampal CA3 pyramidal neurons and tested their sensitivity to ethanol. Concentrations of ethanol as low as 20 mM significantly and reversibly depressed KA EPSCs. Ethanol also inhibited KA currents evoked by direct pressure application of KA in the presence of LY 303070, suggesting that this inhibition was mediated by a postsynaptic action. In contrast, ethanol had no effect on AMPA EPSCs in these cells, even at the highest concentration tested (80 mM). Ethanol significantly inhibited NMDA EPSCs in these neurons, but these responses were less sensitive to ethanol than KA EPSCs. These results suggest that in addition to its well-described depressant effect on NMDA receptor-mediated synaptic transmission, ethanol has an even greater inhibitory effect on glutamatergic synaptic transmission mediated by KA receptors in rat hippocampal CA3 pyramidal neurons.

Glutamate is the principal excitatory neurotransmitter in the mammalian central nervous system. It activates three major classes of ionotropic receptors, which were named based on the agonists initially used to distinguish among these receptors: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate (KA), and *N*-methyl-D-aspartate (NMDA) (Mayer and Westbrook, 1987). There is considerable evidence that ethanol can inhibit the function of the NMDA subtype of glutamate receptor and that this inhibition may underlie at least some of the behavioral and cognitive effects associated with acute ethanol consumption (Lovinger, 1997; Tsai and Coyle, 1998). To that end, intoxicating concentrations of ethanol (10–100 mM) have been shown to inhibit NMDA receptor-mediated synaptic responses in a number of brain regions (Lovinger et al., 1990; Gean, 1992; Nie et al., 1994). Similar concentrations of ethanol have also been shown to antagonize NMDA-evoked currents mediated via both native and recombinant NMDA receptors (see Lovinger, 1997; Faingold et al., 1998, for reviews).

In contrast to the extensive characterization of ethanol inhibition of NMDA receptor function, much less is known about ethanol modulation of non-NMDA receptors. This may be partially due to the fact that most of the initial studies that compared the effects of ethanol on NMDA and non-NMDA receptors reported much more potent ethanol inhibition of NMDA receptor-gated responses (Hoffman et al., 1989; Lovinger et al., 1989). In addition, until recently, appropriate antagonists of either subtype of non-NMDA receptor were not available, making it impossible to pharmacologically isolate AMPA and KA responses in neuronal preparations. It was only with the development of highly selective antagonists of the AMPA subtype of glutamate re-

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ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; aCSF, artificial cerebrospinal fluid; APV, DL-(−)-2-amino-5-phosphonovaleric acid; BMI, bicuculline methiodide; CGP 35348, 3-aminopropyldiethoxymethylphosphinic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EPSC, excitatory postsynaptic current; KA, kainate; LY 303070, (−)-1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-7,8-methylenedioxy-5*H*-2,3,-benzodiazepine; MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; QX-314, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride; TTX, tetrodotoxin.

ceptor (Tarnawa et al., 1993; Zorumski et al., 1993; Paternain et al., 1995; Pelletier et al., 1996) that the first reports of excitatory postsynaptic currents (EPSCs) mediated solely by KA receptors were described (Castillo et al., 1997; Vignes and Collingridge, 1997; Cossart et al., 1998; Frerking et al., 1998; Mulle et al., 1998). These studies have suggested that under most experimental conditions, the "non-NMDA" current characterized in previous work is mediated primarily via AMPA receptors; thus, the sensitivity of native kainate receptors to ethanol remains an open question.

In the present study, we took advantage of the development of AMPA receptor-specific antagonists to pharmacologically isolate synaptic AMPA, KA, and NMDA currents in rat hippocampal CA3 pyramidal neurons and assess their sensitivity to intoxicating concentrations of ethanol.

Materials and Methods

Transverse hippocampal slices (300–400 μm) were prepared from 20- to 40-day-old male Sprague-Dawley rats using a Sorvall tissue chopper or a vibrating tissue slicer (Pelco, Redding, CA). Before recordings, slices were incubated for a minimum of 2 h at 30–32°C in artificial cerebrospinal fluid (aCSF) composed of 124 mM NaCl, 3.3 mM KCl, 2.4 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 10 mM *d*-glucose, and 25.9 mM $NaHCO_3$, saturated with 95% O_2/5% CO_2. Slices were then transferred to a recording chamber maintained at 30–32°C and superfused with aCSF at a constant flow rate of 2 ml/min. Whole-cell patch-clamp recordings were made using glass pipettes pulled on a Flaming/Brown electrode puller (Sutter Instrument Company, Novato, CA). The patch pipette solution contained 130 mM cesium-gluconate, 10 mM $CsCl_2$, 5 mM QX-314 [*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium chloride], 1 mM EGTA, 100 μM $CaCl_2$, 2 mM Mg-ATP, 200 μM Tris-GTP, and 10 mM HEPES. The pH of this solution was 7.25 (adjusted with CsOH), the osmolarity was 285 ± 5 mOsM, and this solution was kept on ice until immediately before use. Glutamatergic synaptic currents were evoked using individual stimuli or brief stimulus trains delivered every 45 to 60 s via bipolar twisted nichrome wire electrodes. Stimulation intensity was set at the lowest level that could evoke stable currents with no failures. AMPA and KA EPSCs were evoked via stimulation of the mossy fiber pathway, within 100 μm of the CA3 pyramidal cell being recorded. NMDA EPSCs were evoked via stimulation of the stratum lacunosum region. In some experiments, KA (100 μM) was applied directly to the soma of CA3 pyramidal cells using a Picospritzer II (General Valve, Fairfield, NJ). These experiments were carried out under visual guidance using an upright microscope equipped with differential interference contrast optics (Nomarski). Drugs used in the pharmacological isolation of synaptic and evoked glutamatergic currents were DL-(−)-2-amino-5-phosphonovaleric acid (APV), 6,7-dinitroquinoxaline-2,3-dione (DNQX), bicuculline methiodide (BMI), citrate-buffered tetrodotoxin (TTX) (all from Sigma, St. Louis, MO), (+)-MK-801 [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate; RBI, Natick, MA], CGP 35348 (3-aminopropyldiethoxymethylphosphinic acid; Novartis, Basel, Switzerland), and LY 303070 [(−)-1-(4-aminophenyl)-3-methylcarbamoyl-4-methyl-7,8-methylenedioxy-5*H*-2,3,-benzodiazepine; Eli-Lilly and Co., Indianapolis, IN]. LY 303070 is the active isomer of the racemate LY 300168 (GYKI-53655). All drugs were made up as stock solutions in DMSO (final total concentration of DMSO, <0.05%), except for APV, BMI, MK-801, and TTX, which were made up as stock solutions in deionized water. A 4 M ethanol solution (AAPER, Shelbyville, KT; diluted in deionized water) was prepared immediately before each experiment from a 100% stock solution kept in a glass storage bottle. These drugs were applied directly to the aCSF via calibrated syringe pumps (Razel, Stanford, CT). Effects of ethanol were quantified as the percent change in current amplitude relative to the mean of control and washout values. Statistical analyses were carried out using ANOVAs followed by the post-hoc Newman-Keuls test or paired *t* tests as indicated, with a minimum level of significance of $p < .05$.

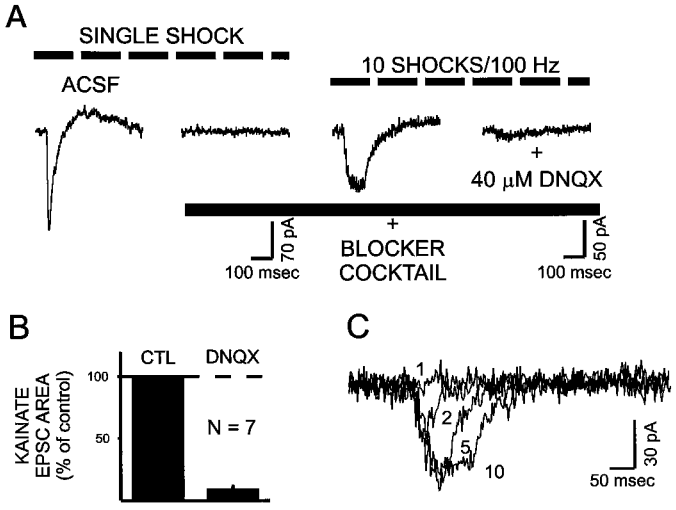

Fig. 1. Pharmacological isolation of synaptic kainate (KA) EPSCs. A, the first two traces are averages of three to five synaptic currents recorded from a CA3 pyramidal neuron in response to single-shock stimulation of the mossy fiber pathway in the absence and presence of a blocker cocktail containing 50 μM APV, 10 μM LY 303070, 20 μM BMI, and 100 μM CGP 35348. The next two traces are averages of four or five EPSCs evoked from the same cell, in the continued presence of the blocker cocktail, using a train of 10 shocks delivered at a frequency of 100 Hz in the absence and presence of 40 μM DNQX. B, summary of the inhibition of KA EPSCs by 40 μM DNQX. C, individual KA EPSCs recorded from a CA3 pyramidal neuron evoked via mossy fiber stimulation using 1, 2, 5, or 10 pulses (as indicated in the figure), delivered at a frequency of 100 Hz. Stimulus artifacts have been deleted from traces for clarity.

Results

Whole-cell recordings were obtained from CA3 pyramidal neurons voltage-clamped between −68 and −72 mV. In normal aCSF, stimulation of the mossy fiber pathway elicited a compound EPSC/inhibitory postsynaptic current in all cells

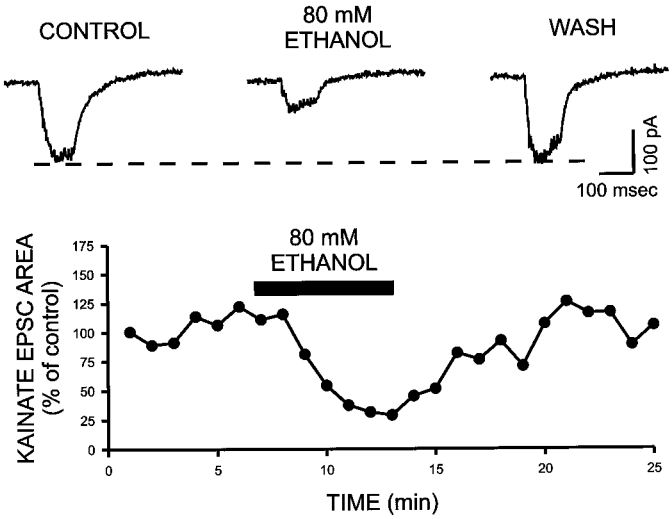

Fig. 2. Time course of ethanol inhibition of KA EPSCs. Traces are averages of four to seven KA EPSCs, recorded in the continuous presence of the standard blocker cocktail, evoked before, during, and after a 6-min application of 80 mM ethanol. Stimulus artifacts have been deleted for clarity.

recorded (Fig. 1A). Bath application of a "blocker cocktail" consisting of 50 μM APV, 10 μM LY 303070, 20 μM BMI, and 100 μM CGP 35348 effectively blocked synaptic transmission in all cells (Fig. 1A). However, as previously reported (Castillo et al., 1997; Vignes and Collingridge, 1997), repetitive stimulation of this pathway in the continued presence of the blocker cocktail invariably resulted in the appearance of a slow inward current. Using a standard stimulation paradigm of 10 shocks delivered at a frequency of 100 Hz every 30 to 45 s, this current had a mean amplitude of 80.8 ± 14.9 pA (n = 29). These responses were not significantly inhibited by raising the concentration of LY 303070 to 40 μM (6.9 ± 4.6% inhibition, n = 4) but were almost completely antagonized by the nonselective AMPA/KA receptor antagonist DNQX (40 μM) (93.1 ± 2.5% inhibition, n = 8). Because this current was evoked in the presence of a maximal concentration of the selective, noncompetitive AMPA receptor antagonist LY 303070 and was antagonized by DNQX, it is thought to be mediated via ionotropic KA receptors (Castillo et al., 1997; Vignes and Collingridge, 1997). Although the standard stimulation protocol used in this study consisted of a train of 10 stimuli, KA EPSCs could be evoked in some cells with as few as 2 stimuli (Fig. 1C).

The ethanol sensitivity of synaptic KA currents was characterized using bath application of 80 mM ethanol (6–8 min), which inhibited KA EPSCs in all cells examined (49.1 ± 5.6% inhibition, n = 10, $p < .01$). This inhibition was apparent within 1 to 2 min and was reversed in most cells after a 5- to 10-min washout (Fig. 2). The ethanol inhibition of KA EPSCs was concentration dependent, with significant inhibition being observed at the lowest ethanol concentration tested (20 mM) (11.0 ± 3.9%; n = 9; $p < .05$).

The ethanol sensitivity of KA EPSCs was compared with that of AMPA and NMDA EPSCs elicited in the same population of CA3 pyramidal cells. AMPA EPSCs were evoked by single-shock stimulation of the mossy fiber pathway in the presence of a blocker cocktail containing 50 μM APV, 20 μM BMI, and 100 μM CGP 35348. This protocol evoked fast, inward currents that were completely inhibited by the selective AMPA receptor antagonist LY 303070 (10 μM) (97.2 ± 4.1% inhibition, n = 6). Bath application of ethanol had no effect on the amplitude of AMPA EPSCs, even at the highest ethanol concentration tested (80 mM, 1.1 ± 3.9% potentiation; n = 6; $p > .05$; Fig. 3). NMDA EPSCs were evoked using single-shock minimal stimulation of the stratum lacunosum in the presence of a blocker cocktail containing 40 μM DNQX, 20 μM BMI, and 100 μM CGP 35348. This protocol evoked inward currents that were completely antagonized by the competitive NMDA receptor antagonist APV (50 μM) (95.6 ± 3.8% inhibition, n = 7). Bath application of ethanol inhibited NMDA EPSCs, but this was significant only at the highest ethanol concentration tested (80 mM; 39.2 ± 2.7% inhibition, $p < .01$, n = 13). Therefore, under our recording conditions, KA EPSCs were more sensitive to ethanol inhibition than synaptic responses mediated by NMDA receptors in CA3 pyramidal neurons ($p < .03$; ANOVA followed by the Newman-Keuls post-hoc test).

Because KA EPSCs were evoked using a different paradigm than that used to elicit AMPA or NMDA EPSCs, it was possible that the greater ethanol sensitivity of KA responses reflected a presynaptic effect of ethanol on glutamate release, rather than a difference at the postsynaptic receptor level. Two additional experiments were carried out to examine this possibility.

In the first experiment, AMPA EPSCs were evoked in the presence of the same blocker cocktail described above using a stimulation protocol identical with that used to evoke KA EPSCs. These responses were evoked with stimulation intensities that did not reliably evoke single responses but did

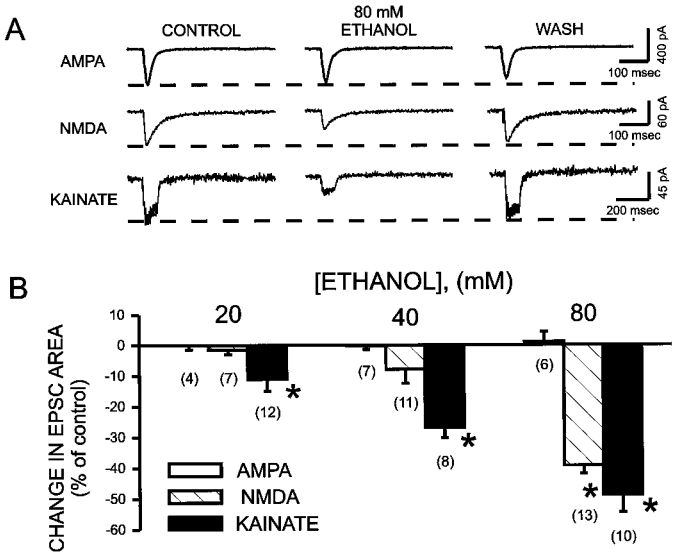

Fig. 3. Concentration dependence of ethanol inhibition of AMPA, NMDA, and KA EPSCs in rat hippocampal CA3 pyramidal neurons. A, representative traces illustrating the effect of 80 mM ethanol on the three subtypes of glutamatergic EPSCs in rat hippocampal CA3 pyramidal neurons. Dashed lines indicate the amplitude of control responses in each case. B, summary of the concentration dependence of ethanol inhibition of the three subtypes of glutamatergic EPSCs (numbers in brackets indicate the number of cells tested under each condition; *$p < .05$). Stimulus artifacts have been deleted from traces for clarity.

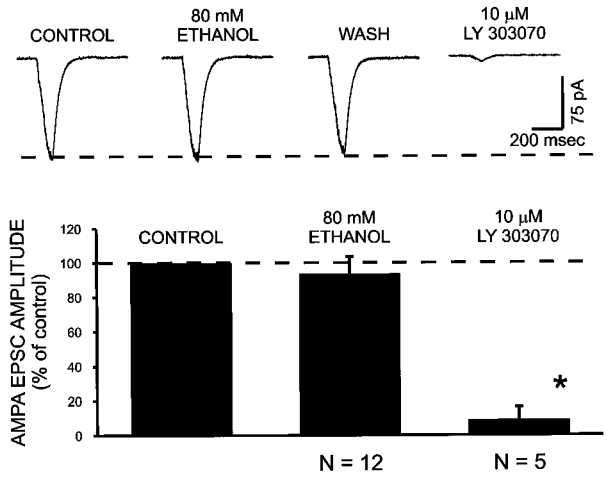

Fig. 4. Effect of ethanol on AMPA EPSCs evoked by the same stimulation train used to generate KA EPSCs. Representative traces illustrating the effect of 80 mM ethanol and 10 μM LY 303070 on synaptic currents evoked using a stimulus train of 10 pulses delivered at a frequency of 100 Hz in the presence of a blocker cocktail containing 50 μM APV, 20 μM BMI, and 100 μM CGP 35348. Stimulus artifacts have been deleted from traces for clarity. Bar graph below traces summarizes the effect of 80 mM ethanol and 10 μM LY 303070 on AMPA EPSCs evoked as described above (*$p < .01$).

result in large inward currents following a train of 10 stimuli delivered at a frequency of 100 Hz (mean amplitude = 117.8 ± 14.0 pA, n = 10). These responses were almost completely antagonized by 10 μM LY 303070 (91.9% ± 7.4%, n = 5) (Fig. 4), suggesting that KA receptors did not contribute significantly to these responses. These AMPA EPSCs, evoked using the same stimulus trains used to evoked KA EPSCs, were not significantly inhibited by 80 mM ethanol (6.4% ± 10.0% inhibition, n = 10) (Fig. 4).

In the second experiment, KA (100 μM) was applied directly to the soma of CA3 pyramidal cells using pressure ejection under visual guidance in the presence of 50 μM APV and 10 μM MK-801 to block NMDA receptors and 0.5 μM TTX to inhibit action potential-dependent synaptic transmission. Brief pressure pulses (5 ms, 5 psi) generated fast inward currents that were completely inhibited by 10 μM LY 303070 and were therefore presumably mediated by the activation of AMPA receptors (Fig. 5A). These currents were not significantly affected by 80 mM ethanol (6.4 ± 4.7% inhibition, n = 7, $p > .05$; Fig. 5A). In approximately 70% of cells tested, increasing the pressure and duration of the KA pulses (20–30 psi, 20–50 ms), in the presence of 10 μM LY 303070, resulted in the appearance of a long-lasting current that was inhibited by 40 μM DNQX (Fig. 5B). The slow kinetics of these responses likely reflects the diffusion of somatically applied KA to the stratum lucidum, where KA receptors are densely expressed (Vignes and Collingridge, 1997; Mulle et al., 1998). Ethanol (80 mM) reversibly inhibited these exogenously activated KA currents (Fig. 5B; 51.2 ± 5.4% inhibition, n = 6, $p < .05$).

Discussion

The results of the present study demonstrate that in rat hippocampal CA3 pyramidal neurons, ethanol significantly inhibits KA receptor-mediated synaptic responses evoked by brief trains of stimuli. In contrast, ethanol had no effect on synaptic currents mediated by AMPA receptor activation, even when evoked with the same stimulation protocol used to generate KA EPSCs. Ethanol also inhibited pharmacologically isolated KA currents evoked by exogenous agonist application, suggesting a postsynaptic mechanism of ethanol inhibition of KA receptor function. Finally, NMDA receptor-gated synaptic currents were also antagonized by ethanol; however, these responses were less sensitive to ethanol than KA EPSCs, under our recording conditions.

Although intoxicating concentrations of ethanol (10–100 mM) effectively inhibit NMDA receptor function (Lovinger, 1997; Faingold et al., 1998; Tsai and Coyle, 1998), ethanol has generally been reported to have much less of an effect on AMPA/KA receptor activity, particularly in rat hippocampus (Lovinger et al., 1989, 1990; Martin et al., 1991). The reason that the potent inhibition of the KA receptor component of non-NMDA EPSCs has not been previously described is now apparent. Non-NMDA receptor-gated responses that have previously been shown to be relatively insensitive to ethanol

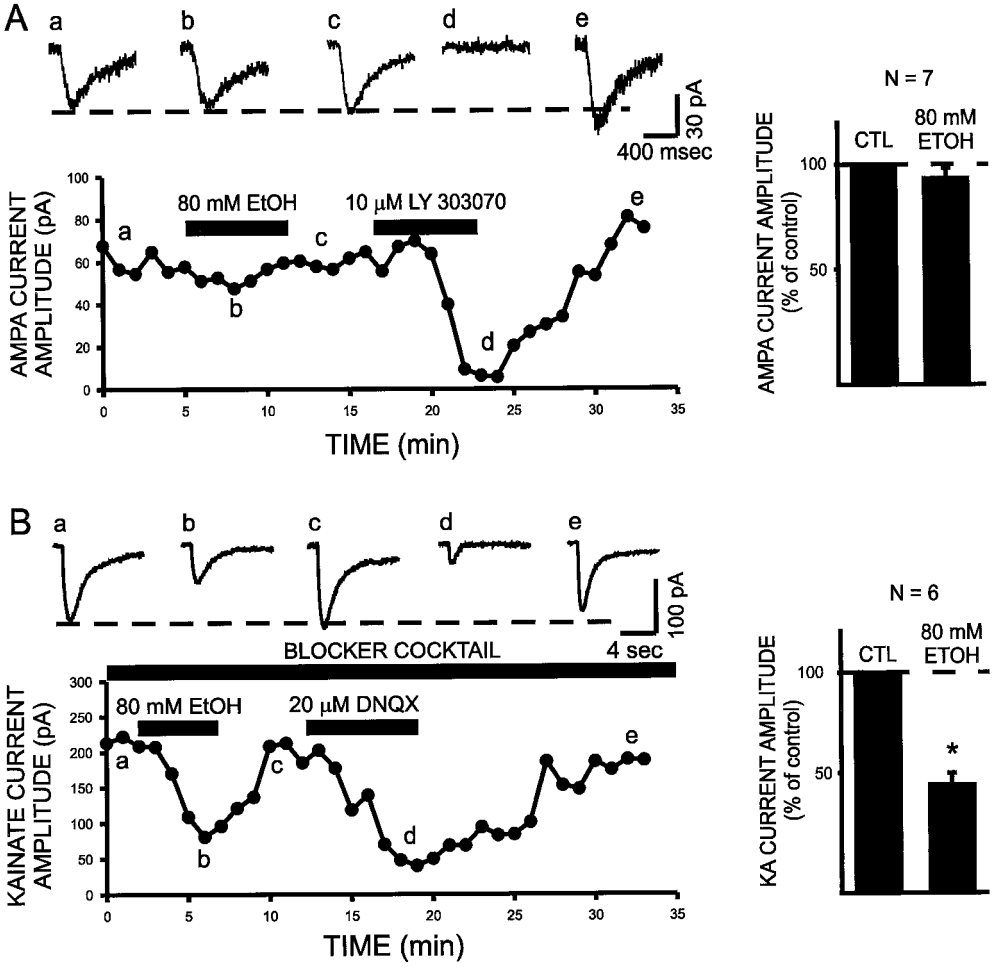

Fig. 5. A, time course of the effect of 80 mM ethanol and 10 μM LY 303070 on currents evoked by brief (<6 ms) pressure application of 100 μM KA to a CA3 pyramidal neuron in the presence of 50 μM APV, 10 μM MK-801, and 0.5 μM TTX. Note that ethanol has no effect on this response and that it is completely and reversibly antagonized by the selective AMPA receptor antagonist LY 303070. Summary of the effect of 80 mM ethanol on AMPA currents is shown at right. B, time course of ethanol and DNQX inhibition of currents evoked by prolonged (30 ms) pressure application of 100 μM KA to a CA3 pyramidal neuron in the presence of 50 μM APV, 10 μM MK-801, 0.5 μM TTX, and the AMPA receptor antagonist LY 303070 (10 μM). The inhibitory effect of 80 mM ethanol on KA currents is summarized at right.

are mediated predominantly, if not entirely, by AMPA receptors, which the present study has demonstrated to be completely unaffected by ethanol at concentrations as high as 80 mM. Synaptic KA receptors are only activated by relatively intense stimulation paradigms and are not even present in some neuronal populations (Castillo et al., 1997; Vignes and Collingridge, 1997; Cossart et al., 1998; Frerking et al., 1998). It should be noted that in the nucleus accumbens, ethanol has been reported to inhibit non-NMDA receptor-mediated synaptic responses at concentrations similar to those observed in this study (Nie et al., 1993). Although KA receptor subunits are abundantly expressed in this brain region (Bischoff et al., 1997; Wullner et al., 1997), it remains to be determined whether these receptors contribute appreciably to glutamatergic synaptic transmission in these cells.

Although this is the first report of ethanol inhibition of synaptic KA responses, other studies have tested the effects of ethanol on recombinant KA receptors (Dildy-Mayfield and Harris, 1995; Valenzuela et al., 1998a). In these previous studies, ethanol significantly inhibited KA receptor function, although less potently than the inhibition of synaptic KA responses observed in the present study. Moreover, these studies also reported significant ethanol inhibition of recombinant AMPA receptor activity, with a potency similar to that of recombinant KA receptor inhibition. Another recent study on pharmacologically isolated AMPA and KA currents in cerebellar granule cells also suggested a lack of selectivity in the depressant effects of ethanol on these currents (Valenzuela et al., 1998b). Although these findings support the hypothesis that KA receptors may be sensitive to intoxicating concentrations of ethanol, they do not provide an explanation for the marked differential ethanol sensitivity of synaptic AMPA and KA receptors in rat hippocampal CA3 pyramidal neurons.

There are a number of factors that could account for the differences in ethanol sensitivity of AMPA and KA responses observed in the present study and the lack of such differences in previous studies; these factors include differences in the subunit composition of KA receptors in different brain regions or differences in the assembly or posttranslational modification of these receptors. In fact, hippocampal KA receptors display a number of unique properties that distinguish them from recombinant KA receptors or native KA receptors expressed in other brain regions. For example, KA receptors in cultured hippocampal neurons display much slower and less complete desensitization than recombinant KA receptors (Wilding and Huettner, 1997). In addition, the lectin concanavalin A reduces peak KA currents in hippocampal pyramidal neurons (Wilding and Huettner, 1997), whereas this compound has been shown to slow desensitization and often increase peak KA currents in recombinant (Egebjerg et al., 1991; Partin et al., 1993) and native KA receptors (Wong and Mayer, 1993; Valenzuela et al.,1998b). Further studies will clearly be needed to delineate the mechanisms underlying these distinct properties of hippocampal KA receptors.

In summary, we have shown that ethanol potently attenuates KA receptor-mediated synaptic transmission in rat hippocampal CA3 pyramidal neurons and that these effects are observed at lower ethanol concentrations than are required to elicit the well-characterized inhibitory effect of ethanol on NMDA receptor function. The physiological role of synaptic kainate receptors is largely unknown, so it is difficult to evaluate the significance of KA receptor inhibition vis a vis the behavioral and cognitive changes associated with ethanol intoxication. One possible consequence of ethanol inhibition of KA receptor-gated synaptic transmission may be to contribute to the anticonvulsant actions of this drug. Acute ethanol administration can significantly inhibit seizure activity in a variety of animal models (Cohen et al., 1993; Kleinrok et al., 1993). The CA3 region of the hippocampus is known to play an integral role in limbic seizure generation (Ben-Ari, 1985; Barbarosie and Avoli, 1997). Moreover, deletion of the GluR6 subunit, which eliminates KA EPSCs in CA3 pyramidal neurons, dramatically decreases KA-stimulated seizures in mice (Mulle et al., 1998). Therefore, ethanol inhibition of KA receptor-mediated excitation in CA3 pyramidal neurons would likely reduce seizure activity in this brain region. Several recent studies have demonstrated that KA receptors in the CA1 region of the hippocampus may play an important role in regulating γ-aminobutyric acid (Cossart et al., 1998; Frerking et al., 1998; Rodriguez-Moreno et al., 1998) and glutamate (Chittajallu et al., 1996) release, which further expands the types of responses that might be affected by ethanol. Further studies will be required to determine whether these receptors, as well as KA receptors in other brain regions, possess the same ethanol sensitivity as the KA receptors expressed in CA3 pyramidal neurons.

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