

Ethanol differentially enhances hippocampal GABA_A receptor-mediated responses in PKC γ and PKC ϵ null mice.

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Abbreviations: PKC $\gamma^{+/+}$ and PKC $\gamma^{-/-}$, wild-type and null mutant mouse lines for the gamma-protein kinase C isoform; PKC $\epsilon^{+/+}$ and PKC $\epsilon^{-/-}$, wild-type and null mutant mouse lines for the epsilon protein kinase C isoform; LORR, loss of righting reflex; IPSC and EPSC, inhibitory and excitatory post-synaptic current; NMDA, N-methyl-D-aspartate;

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxalone propionic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; APV, DL (-)-2-amino-5-phosphonovaleric acid; CGP 35348, (3-Aminopropyl)(diethoxymethyl) phosphinic acid; BEC, blood ethanol concentration

Abstract

Ethanol intoxication results partly from actions of ethanol at specific ligand-gated ion channels. One such channel is the GABA_A receptor complex, although ethanol's effects on GABA_A receptors are variable. For example, we found that hippocampal neurons from selectively bred mice and rats with high hypnotic sensitivity to ethanol have increased GABA_A receptor-mediated synaptic responses during acute ethanol treatment compared with mice and rats that display low behavioral sensitivity to ethanol. Here we investigate whether specific protein kinase C (PKC) isozymes modulate hypnotic and GABA_A receptor sensitivity to ethanol. We examined acute effects of ethanol on GABA_A receptor-mediated inhibitory post-synaptic currents (IPSCs) in mice lacking either PKC γ (PKC γ ^{-/-}) or PKC ϵ (PKC ϵ ^{-/-}) isozymes and compared the results to those from corresponding wild-type littermates (PKC γ ^{+/+} and PKC ϵ ^{+/+}). GABA_A receptor-mediated IPSCs were evoked in CA1 pyramidal neurons by electrical stimulation in stratum pyramidale, and the responses were recorded in voltage-clamp mode using whole-cell patch recording techniques. Ethanol (80 mM) enhanced the IPSC response amplitude and area in PKC γ ^{+/+} mice, but not in the PKC γ ^{-/-} mice. In contrast, ethanol markedly potentiated IPSCs in the PKC ϵ ^{-/-} mice, but not in PKC ϵ ^{+/+} littermates. There was a positive correlation between ethanol potentiation of IPSCs and the ethanol-induced loss of righting reflex such that mice with larger ethanol-induced increases in GABA_A receptor-mediated IPSCs, also had higher hypnotic sensitivity to ethanol. These results suggest that PKC γ and PKC ϵ signaling pathways reciprocally modulate both ethanol enhancement of GABA_A receptor function and hypnotic sensitivity to ethanol.

The mechanisms of ethanol intoxication are complex and involve many regions of the brain. Although alcohol was once thought to act non-selectively to modify lipid mobility in neuronal plasma membranes, it is now clear that ethanol interacts at specific neuronal proteins, including some voltage- and ligand-gated ion channels (Lovinger, 1997; Mihic, 1999). In general, acute ethanol treatment decreases excitation via suppression of an NMDA-activated current, and increases inhibition by enhancing GABA_A receptor-mediated conductance, although there is large variability in the reported effects of ethanol on these and other receptor-channel complexes (Crews et al., 1996). The GABA_A receptor complex is the primary mediator of fast inhibitory neurotransmission in the central nervous system and is an important target of anesthetic compounds (Mihic et al., 1994; Harris, 1999). Ethanol has a considerable range of effects on GABA_A receptor-mediated responses. For example, intoxicating concentrations of ethanol enhance GABA_A receptor-mediated Cl⁻ flux in brain synaptosomal or microsac preparations (Allan and Harris, 1986) and in cultured neurons (Mehta and Ticku, 1994). Electrophysiological studies have shown that ethanol increases GABA_A receptor function in some brain preparations (Aguayo, 1990; Reynolds et al., 1992; Weiner et al., 1997a; Soldo et al., 1998; Poelchen et al., 2000; Nie et al., 2000), but reports from other studies have found no significant ethanol potentiation (Osmanovic and Shefner, 1990; White et al., 1990).

Our laboratory previously identified a GABAergic synaptic region on CA1 pyramidal cells at, or near, the cell soma that is consistently potentiated by ethanol (Weiner, et al., 1997a); while the outer dendrites have little or no ethanol modulation. This observation might account for much of the confusion over ethanol's action in the

CA1 hippocampal region in brain slices. One possible reason for these ethanol-sensitive and -insensitive areas is that various synapses contain receptors that differ in subunit composition and thereby display differences in ethanol sensitivity. Genetic factors are also involved in mediating ethanol sensitivity of GABA_A receptors. Studies of acute ethanol treatment on GABA or muscimol-stimulated Cl⁻ flux in isolated brain microsacs (Allan and Harris, 1986) and GABA_A receptor-mediated inhibitory post-synaptic currents (IPSCs; Poelchen et al., 2000) both show robust differences in lines of mice and rats selectively bred for differences in initial sensitivity to the hypnotic effects of ethanol, as measured by the duration of the ethanol-induced loss of the righting reflex (LORR or sleep time). Ethanol (80 mM) significantly increases GABA_A responses in CA1 pyramidal cells in hippocampal brain slices from High Alcohol Sensitive (HAS₁ and HAS₂) rat and Inbred Long Sleep (ILS) mouse lines that are highly sensitive to the hypnotic effects of ethanol (Draski et al., 1992). Moreover, Low Alcohol Sensitive (LAS₁ and LAS₂) rats and the Inbred Short Sleep (ISS) mice, the corresponding selected lines that are relatively insensitive to ethanol, do not show a significant change in pyramidal cell GABA_A IPSCs after 80 mM ethanol treatment. These findings provide strong evidence that GABA_A receptors are a specific target of ethanol action that, at least in part, mediates the hypnotic sensitivity to ethanol. The findings also suggest that there are genetic factors that modulate GABA_A receptor and hypnotic sensitivity in parallel.

Studies in our laboratories (Messing et al., 1991; Weiner et al., 1997b) and others (Mironov and Hermann, 1996; Gordon et al., 1997; but see Deitrich et al. 1989) suggest that protein kinase C (PKC) is involved in responses to ethanol. Previous studies of PKC γ and PKC ϵ wild-type and null mutant mice suggest a role for these PKC isozymes

in regulating initial sensitivity to the hypnotic effects of ethanol and ethanol potentiation of GABA-ergic function as measured by Cl^- flux in brain microsac preparations from these animals (Harris et al., 1995; Hodge et al., 1999; Bowers et al., 1999). $\text{PKC}\gamma^{-/-}$ mice demonstrate reduced sensitivity to the hypnotic effects of ethanol compared to $\text{PKC}\gamma^{+/+}$ wild-type controls and show reduced ethanol potentiation of muscimol-stimulated Cl^- flux in microsacs prepared from $\text{PKC}\gamma^{-/-}$ cerebellum and cerebral cortex (Harris et al., 1995). In contrast, ethanol induced sleep time and ethanol potentiation of muscimol-stimulated Cl^- are greater in the $\text{PKC}\epsilon$ null mutants than in the wild-type littermates (Hodge et al., 1999). Because changes in PKC activity might be one of the mechanisms regulating the enhancement of the hippocampal GABA_A receptor-mediated IPSCs by ethanol, we tested animals lacking $\text{PKC}\gamma$ or $\text{PKC}\epsilon$ to determine if either or both of these PKC isozymes affect ethanol action at GABAergic synapses.

Material and Methods

Animals. Adult (3-6 month old) mice were used from two lines of animals having a mutation in one of the PKC isozymes, epsilon or gamma. Male and female PKC γ null mutant (PKC $\gamma^{-/-}$) and PKC γ wild-type (PKC $\gamma^{+/+}$) mice were derived from a 129/SvevTac x C57BL/6J background as described previously (Harris et al., 1995; Bowers et al., 1999). Male and female PKC ϵ null mutant (PKC $\epsilon^{-/-}$) and PKC ϵ wild-type (PKC $\epsilon^{+/+}$) mice were derived from a 129SvJae x C57BL/6J background (Hodge et al., 1999).

Sleep times. Mice were injected with a single intraperitoneal (i.p.) dose of 3.5 g/kg ethanol (20% w/v), and were placed in a V-shaped trough after becoming ataxic. They were then monitored for the time required to regain their righting response three times in a 30-s time period. The time between initial loss and the recovery of the righting reflex, was recorded as the “sleep time” or duration of the loss of righting reflex (LORR). At the time of righting, a blood sample from the retro-orbital sinus was taken and the concentration of ethanol determined as described previously (Harris et al., 1995).

Slice preparation, storage and recording bath conditions. Mice were killed by cervical dislocation, and their brains were rapidly removed and immersed in either ice-cold artificial cerebrospinal fluid (aCSF) or high-sucrose buffer for 60 sec to cool the interior of the brain. The aCSF consisted of: 126 mM NaCl, 3.0 mM KCl, 1.5 mM MgCl₂, 2.4 mM CaCl₂, 1.2 mM NaH₂PO₄, 11 mM D-glucose, and 25.9 mM NaHCO₃. The high-sucrose buffer contained 87 mM NaCl, 2.5 mM KCl, 7 mM MgCl₂, 0.5 mM CaCl₂, 1.25 mM NaH₂PO₄, 25 mM D-glucose, 75 mM sucrose, and 25 mM NaHCO₃ (Geiger and Jonas, 2000). The buffers were continuously oxygenated with 95% O₂ / 5%

CO₂. After removing one or both hippocampi from the brain, 400 μm thick transverse slices were made using a Sorvall tissue chopper (Newtown, CT). The slices were temporarily submerged in ice-cold aCSF or high-sucrose buffer until all the slices were collected, and then were transferred to individual compartments in a storage system that was constantly perfused with 95% O₂ / 5% CO₂ (Proctor and Dunwiddie, 1999; Weiner, 2002) containing either aCSF or a 50:50 mix of aCSF and high-sucrose buffer at 32 to 33° C. Slices were stored in this condition for 1 to 10 h and then transferred via large-mouth Pasteur pipette to a nylon net in a recording chamber (0.5 ml volume) and constantly superfused with bubbled aCSF at a rate of 2.0 mL/min at 32 to 33°C.

Electrophysiological recording. Patch microelectrodes were constructed from borosilicate glass capillary tubes (1.5 mm O.D., 0.86 mm I.D.; Sutter Instrument Co., Novato, CA), and pulled apart under a heated platinum/iridium filament (model P-87 micro-pipette puller; Sutter Instrument Co.) to a tip size of approximately 1 μm in diameter having resistances of 6 to 9 MΩ when filled with a K⁺-glucose internal solution containing 130 mM K-glucose, 0.8 mM KCl, 0.1 mM CaCl₂, 2.0 mM MgCl₂, 1.0 mM EGTA, 10.0 mM HEPES, 2.0 mM Mg-ATP, and 0.3 mM Na-GTP, adjusted to pH 7.3 with KOH, 290 mOsm. CA1 pyramidal neurons were recorded in the whole-cell configuration. The cells were voltage-clamped to -60 mV (corrected for the liquid-junction potential) from the normal resting membrane potential (-65 to -70 mV) to reduce any contamination of the GABA_A IPSC response by small, slow GABA_B currents. GABA_A receptor-mediated IPSC responses were evoked (200 μs, 4–10 V pulse) with a bi-polar tungsten, stimulating electrode at 30 – 60 sec intervals positioned in the stratum pyramidale within 300 μm of the whole-cell recording electrode. This stimulation-

recording paradigm evoked synaptic responses predominantly from proximal inputs (i.e., GABA_A responses from interneurons that synapse on or near the soma of the recorded pyramidal cell), which were modulated by ethanol in several rat and mouse lines (Weiner et al., 1997a; Poelchen et al., 2000). All chemicals used to prepare electrode solutions were purchased from Fluka Chemical Corp (Milwaukee, WI).

Drugs. To pharmacologically isolate GABA_A receptor-mediated inhibitory post-synaptic currents (IPSCs), 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μ M final chamber bath concentration) and DL-(-)-2-amino-5-phosphonovaleric acid (APV; 50 μ M) were added to the superfusate to block α -amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) excitatory post-synaptic responses (EPSCs), respectively. In some experiments, the GABA_B receptor antagonist (CGP 35348) was added to facilitate measuring GABA_A receptor-mediated responses. Except as noted elsewhere, all drugs were purchased from Sigma Chemical Co. (St. Louis, MO). The drugs were prepared as 50 to 100 fold concentrates in 12 cc syringes (Monojet, polypropylene; Sherwood Medical, St. Louis, MO) and were added to the superfusate via syringe pumps (Razel Scientific Instruments, Stamford, CT). Ethanol was diluted to a 5.0 M working solution with deionized water from a 95% stock solution and stored cold in sealed glass bottles prior to loading into a 12 cc syringe. For these studies, a concentration of 80 mM ethanol was used since it approximates the average blood and brain levels (360 mg%) measured when mice regain their righting reflex (Draski et al., 1992).

Data analysis. After a 20- to 30-min superfusion with DNQX and APV, the stimulus intensity was adjusted to produce a GABA_A receptor-mediated IPSC of 20 to

120 pA peak amplitude. For each cell tested, the peak amplitude and the area under the curve of the GABA_A response were measured before, during and following ethanol treatment (80 mM) to determine the effect of ethanol on the GABA_A response. The percent change in the amplitude and the area under the curve for each recorded cell was determined during the 5- to 15-min interval after the start of ethanol superfusion. These results were compared to the mean value of the control and washout periods (the washout measurement was begun 20 to 30 min after the end of the ethanol treatment). Membrane resistance and holding current were also monitored for each cell.

Statistical analysis. Sleep time and electrophysiological data were analyzed using two-tailed Student's paired and un-paired *t* tests, or two-way analysis of variance as indicated. Pearson's product-moment correlation analysis was done to evaluate the association between ethanol-induced sleep time values and the effect of ethanol on GABA_A IPSC enhancement. In all tests, a *P* value less than 0.05 was considered to be statistically significant.

Results

The first set of experiments was designed to compare ethanol-induced behavioral effects among the four groups of mice: PKC γ wild-type (PKC $\gamma^{+/+}$), PKC γ null mutant (PKC $\gamma^{-/-}$), PKC ϵ wild-type (PKC $\epsilon^{+/+}$), and PKC ϵ null mutant (PKC $\epsilon^{-/-}$). The duration of LORR was measured after intraperitoneal-administration of 3.5 g/kg ethanol. PKC $\gamma^{-/-}$ mice were significantly less sensitive to ethanol than PKC $\gamma^{+/+}$ littermate controls (Fig. 1A). In contrast, PKC $\epsilon^{-/-}$ mice were significantly more sensitive to ethanol than their wild-type (PKC $\epsilon^{+/+}$) littermates (Fig. 1B). Within the two wild-type genotypes, PKC $\gamma^{+/+}$ and PKC $\epsilon^{+/+}$, LORR responses were also significantly different. The PKC γ and PKC ϵ lines are derived from two different 129 inbred strains crossed to C57BL/6J; i.e., 129/SvevTac x C57BL/6J and 129SvJae x C57BL/6J, respectively. There is considerable genetic variation among the 129 substrains (Simpson et al., 1997); therefore it is not unexpected that phenotypic differences were observed between the respective wild-type mice due to the two different background 129 strains used. Several studies have reported differences in behavior among the 129 strains (for review, see Simpson et al., 1997).

We also analyzed each genotype for their blood ethanol concentration (BEC) at the time of regaining the righting reflex, excluding values from mice that did not lose the reflex after ethanol injection (Table 1). This provides an indication of changes in CNS sensitivity as opposed to alterations in metabolic rates of ethanol elimination. Previous reports have shown that ethanol elimination rates do not differ between mutant and wild-type mice for either the PKC γ (Harris et al., 1995) or the PKC ϵ mice (Hodge et al., 1999). Within each line of mice, the genotypes with the shortest sleep-time, PKC $\gamma^{-/-}$ and PKC $\epsilon^{+/+}$, awoke with higher BECs than the corresponding genotypes of each line

indicating that the differences in sleep times between the genotypes within a line were not simply due to differences in the rate of ethanol metabolism.

Electrophysiological recordings were made from CA1 pyramidal neurons in hippocampal slices from PKC γ and PKC ϵ mutant mice and from their wild-type littermates. After blocking glutamatergic responses with DNQX and APV, the remaining responses were mediated primarily via GABA $_A$ receptors, but in some instances there was a small, late component on the falling phase of the IPSC that could be blocked by addition of CGP 55845 (Tocris Cookson Inc., Ballwin, MO), a selective GABA $_B$ receptor antagonist (data not shown). As this late component was small and did not overlap significantly with the peak of the GABA $_A$ response, CGP 55845 was not normally used in these recordings. Ethanol enhanced the amplitude and area under the curve for GABA $_A$ receptor-mediated IPSCs in slices from the PKC $\gamma^{+/+}$ mice (Fig. 2A), but no ethanol effect was observed in slices from PKC $\gamma^{-/-}$ mice (Fig 2B). In contrast, IPSCs in slices from PKC $\epsilon^{-/-}$ mice (Fig 2D) showed greater enhancement during ethanol application than did IPSCs measured in slices from their wild-type littermates (Fig 2C). These representative tracings showed that enhancement of the peak GABA $_A$ IPSC response appeared in PKC $\gamma^{+/+}$ and PKC $\epsilon^{-/-}$ slices within 5 min after initial exposure to ethanol (Fig. 3A and B) whereas there was no significant enhancement of the peak response in PKC $\gamma^{-/-}$ and PKC $\epsilon^{+/+}$ slices. There was no detectable short-term desensitization or tolerance during the ethanol application, and recovery to baseline required approximately 5 to 10 min following the completion of the ethanol application. The GABA $_A$ response in the ethanol sensitive animals was similar to what we have previously reported in other lines of rats and mice, requiring 3 to 8 min of ethanol

superfusion to obtain maximal enhancement of the IPSC (Poelchen et al., 2000).

Analysis of the IPSC peak amplitude on all tested cells revealed that PKC γ ^{-/-} neurons are much less sensitive to ethanol enhancement of GABA_A receptor-mediated IPSCs than are their wild-type control neurons (Fig. 4A), whereas PKC ϵ ^{-/-} neurons are more sensitive to ethanol modulation than the corresponding wild-type neurons (Fig. 4B).

We also measured the holding current, the cell membrane resistance, and the area under the curve for GABA_A IPSCs during ethanol application (Table 2). In the presence of ethanol, there was a small (~10 pA) increase in the holding current in neurons from both lines of wild-type mice and for PKC ϵ null mice; only the holding current in neurons from PKC γ null mutants was not significantly affected by ethanol. Membrane resistance was not significantly altered by ethanol in any of the slices, but differential effects of ethanol on measurements of the area under the GABA_A IPSC curve closely paralleled increases in the peak amplitude measurements.

To examine the relationship between ethanol's effect on behavior and GABA_A IPSCs, a regression analysis was conducted (Fig. 5). There was a strong, significant correlation ($r^2 = 0.95$; $P < 0.05$) between the enhancing effect of ethanol on the GABA_A IPSC and the duration of the LORR.

Discussion

We have previously reported that increases in the PKC activity enhance the ethanol sensitivity of GABA_A receptors in rat hippocampal pyramidal neurons (Weiner et al., 1997b). Mice deficient in one of two isozymes of PKC, PKC γ and PKC ϵ , were examined for the role of these isozymes in the ethanol-induced modulation of hippocampal GABA_A receptor-mediated IPSCs. Slices from these mice had different electrophysiological responses to ethanol (80 mM), suggesting that ethanol sensitivity of GABA_A synapses is modulated by PKC γ and PKC ϵ . Because there is generally little difference between PKC isozymes in *in vitro* substrate specificity (Mochly-Rosen and Gordon, 1998), differences in subcellular localization due to protein-protein interactions, or tissue distribution, and differences in responses to second messengers are likely to account for these differential effects. Within the same cells, the subcellular distribution of these enzymes is likely to be different because activated PKC ϵ binds to F-actin (Prekeris et al., 1996) and the cotamer protein β' -COP (RACK2) (Csukai et al., 1997), but PKC γ does not. In the CA1 region of the rat hippocampus, the tissue distribution of these isozymes also differs. There PKC γ is located in the cell body, dendrites, and dendritic spines of pyramidal neurons (Kose et al., 1990). PKC γ might also associate with receptor subunits in hippocampus because in rat cerebral cortex it can be co-immunoprecipitated with GABA_A α 1 and α 4 subunits (Kumar et al., 2002). In contrast, PKC ϵ is found in rat CA1 stratum radiatum near synaptic vesicles, but not in postsynaptic dendrites or pyramidal cell bodies (Saito et al., 1993). In addition to differences in hippocampal localization, PKC γ can be activated by calcium whereas PKC ϵ cannot.

The findings of the present study are in agreement with our previous results (Poelchen et al., 2000) that demonstrate a relationship between behavioral sensitivity to ethanol in selected lines of rodents and *in vitro* ethanol sensitivity of hippocampal GABAergic synapses. The strong correlation between ethanol's effects on GABA_A receptor-mediated IPSCs and sleep times in these PKC wild-type and mutant mice suggests that modulation of GABA_A receptors by these isozymes contributes to the hypnotic effects of ethanol. The correlation between hypnotic sensitivity and hippocampal IPSCs may not reflect a cause and effect relationship since the hippocampus is not thought to mediate hypnotic responses. However, these PKC isozymes are expressed in the cerebral cortex which is important for wakefulness, and previous Cl⁻ flux studies have shown enhanced ethanol modulation of GABA_A receptors in PKC $\epsilon^{-/-}$ mice (Hodge et al., 1999) and reduced modulation in PKC $\gamma^{-/-}$ mice (Harris et al., 1995) in cortical tissue. Therefore, this correlation between behavioral sensitivity and GABA_A receptor-mediated responses to hypnotic concentrations of ethanol is likely to hold for other brain areas such as the cerebral cortex, which play a role in hypnotic responses to drugs. The fact that wild-type littermates of the two mouse lines exhibited different responses to ethanol in both assays suggests that within these two lines there are parallel polymorphisms in other genes that regulate GABA_A receptors and sleep time. Several quantitative trait loci for hypnotic sensitivity to ethanol have been identified in mice using recombinant inbred strains (LS X SS and C57Bl/6J X DBA/2J) (Markel et al., 1997; Browman and Crabbe, 2000). It is likely that allelic differences between 129SvJae and 129/SvevTac lines account for the differences in ethanol sleep time and synaptic function that we observed in the wild-type mice.

A limitation of our study is that we used conventional knockout mice, so it is possible that the phenotypes resulted from a developmental change rather than absence of PKC γ or PKC ϵ signaling in adult tissues. As previously reported, the selective PKC ϵ inhibitor peptide, ϵ V1-2, enhances ethanol and flunitrazepam potentiation of muscimol-stimulated GABA $_A$ receptor function in tissue from wild-type but not PKC ϵ null mice (Hodge et al., 1999), suggesting that it is the absence of PKC ϵ in adult neurons and not altered development that accounts for enhanced GABA $_A$ receptor sensitivity in PKC ϵ null mice. It was recently reported that transgenic restoration of PKC ϵ , by means of a tetracycline regulated prion promoter that drives expression mainly in neurons, rescues the altered sleep time phenotype in PKC ϵ null mice (Choi et al., 2002). This finding suggests that the changes in hypnotic sensitivity in PKC ϵ null mice are unlikely to be due to altered development. Similar studies have not been performed in PKC γ null mice.

The relationship that we found between sleep time and modulation of GABA $_A$ receptor-mediated IPSCs in these mice is also true for ethanol enhancement of muscimol-stimulated GABA $_A$ mediated Cl $^-$ flux measurements in brain microsome preparations (Hodge et al., 1999; Harris et al., 1995). In cerebellar tissue from PKC $\gamma^{-/-}$ mice, ethanol potentiation of muscimol-stimulated Cl $^-$ flux was completely eliminated and was significantly reduced in cortical tissue compared to PKC $\gamma^{+/+}$ controls. In contrast, in PKC $\epsilon^{-/-}$ mice, ethanol enhancement of muscimol-stimulated Cl $^-$ flux in frontal cortex was significantly greater than in PKC $\epsilon^{+/+}$ controls. Therefore, the pattern of ethanol potentiation of chloride flux in these knockout mice is in agreement with the present results showing differential modulation by ethanol of hippocampal GABA $_A$ IPSCs.

There is considerable variability in ethanol sensitivity in studies of ethanol effects on GABA_A responses in non-selected lines of rodents, even in studies of what would seem to be the same receptors in the same population of cells (Proctor et al., 1992a; Proctor et al., 1992b; Wan et al., 1996; Weiner et al., 1997a; Peoples and Weight, 1999). This suggests that ethanol sensitivity must depend on a specific combination of factors. One such variable is the subpopulation of GABA_A receptors activated by synaptic stimulation. Previous work by Pearce and colleagues (Pearce, 1993) demonstrated that there are populations of GABA_A synapses on CA1 pyramidal neurons that differ in a variety of respects, including their kinetic properties, and sensitivity to pharmacological agents such as furosemide. Our studies in Sprague-Dawley rats have shown that distal GABA_A-mediated IPSCs are less sensitive to ethanol than are proximal IPSCs (Weiner et al., 1997a). In the present study, PKC γ and PKC ϵ related changes in ethanol sensitivity were limited to proximal IPSCs and there were no significant differences in distal GABA_A-mediated responses in these mice (data not shown). Therefore it is likely that other factors besides PKC γ and PKC ϵ account for the differential sensitivity of proximal and distal GABA-ergic synapses to ethanol.

In summary, the present results extend our earlier findings that selected rat and mouse lines that are behaviorally more sensitive to ethanol have greater ethanol-induced enhancement of GABA_A receptor-mediated responses than do animals that are less sensitive to the behavioral effects of ethanol (Poelchen et al., 2000). The present data reveal a strong correlation between the sedative/hypnotic sensitivity to ethanol and the enhancement of GABA_A receptor-mediated responses during ethanol treatment in the PKC null mutants and their wild-type mouse lines. These results also suggest that PKC is

involved in the mechanism that underlies the modulation of the GABA_A response in animals that are more sensitive to the behavioral effects of ethanol.

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Figure Legends

Fig. 1. Duration of the ethanol-induced loss of righting reflex (LORR) following a single intraperitoneal injection of 3.5 g/kg ethanol in (A) PKC γ wild-type (PKC $\gamma^{+/+}$) and null mice (PKC $\gamma^{-/-}$) and (B) PKC ϵ wild-type (PKC $\epsilon^{+/+}$) and null mice (PKC $\epsilon^{-/-}$). The numbers at the base of each bar represent the number of animals examined from each group.

* $p < 0.05$, unpaired two-tailed t -test.

Fig. 2. Effect of bath superfusion with 80 mM ethanol on GABA $_A$ IPSCs. GABA $_A$ receptor-mediated responses were initiated by proximal stimulation in the presence of DNQX and APV. Each set of traces was obtained from a single pyramidal neuron. Averaged responses from 8 to 15 traces were recorded under control conditions (Con), in the presence of 80 mM ethanol (EtOH), and after ethanol washout (Wash). Scale bars: Horizontal = 50 ms; Vertical = 10 pA (A, B), 5 pA (C, D).

Fig. 3. Representative plots showing the time course of the ethanol effect on GABA $_A$ IPSC peak amplitudes measured in single pyramidal neurons from (A) PKC $\gamma^{+/+}$ and PKC $\gamma^{-/-}$ mice and (B) PKC $\epsilon^{+/+}$ and PKC $\epsilon^{-/-}$ mice.

Fig. 4. Effect of ethanol on the peak amplitude of the GABA $_A$ IPSC response in pyramidal neurons from (A) PKC $\gamma^{+/+}$ and PKC $\gamma^{-/-}$ mice and (B) PKC $\epsilon^{+/+}$ and PKC $\epsilon^{-/-}$ mice. Data are mean \pm s.e. values and are expressed relative to the peak IPSC amplitude measured prior to bath application of 80 mM ethanol. The number of cells recorded from

each mouse line is shown at the bottom of each bar. $*p < 0.05$, unpaired two-tailed t -test.

Fig 5. Linear regression analysis of enhancement of GABA_A receptor-mediated IPSC responses (peak amplitude) by ethanol (80 mM) and the duration of the LORR after ethanol injection (3.5 mg/gm). The mice tested for LORR were a subgroup of animals that were used to obtain the hippocampal slices for the electrophysiological experiments. The number of animals tested for sedative-hypnotic effects, followed by the number of cells recorded from these and additional mice are: PKC $\gamma^{+/+}$ 11, 26; PKC $\gamma^{-/-}$ 7, 32; PKC $\epsilon^{+/+}$ 5, 33; PKC $\epsilon^{-/-}$ 5, 27.

TABLE 1

Blood ethanol concentration (mean \pm s.e.m.) upon recovery of LORR.

	Mouse line	
Genotype	PKC γ	PKC ϵ
+/+	315 \pm 13.1, n=10	347 \pm 9.3, n=12
-/-	*349 \pm 7.9, n=7	**297 \pm 9.6, n=10

* $p = 0.07$ compared with PKC $\gamma^{+/+}$, ** $p < 0.001$ compared with PKC $\epsilon^{+/+}$

TABLE 2

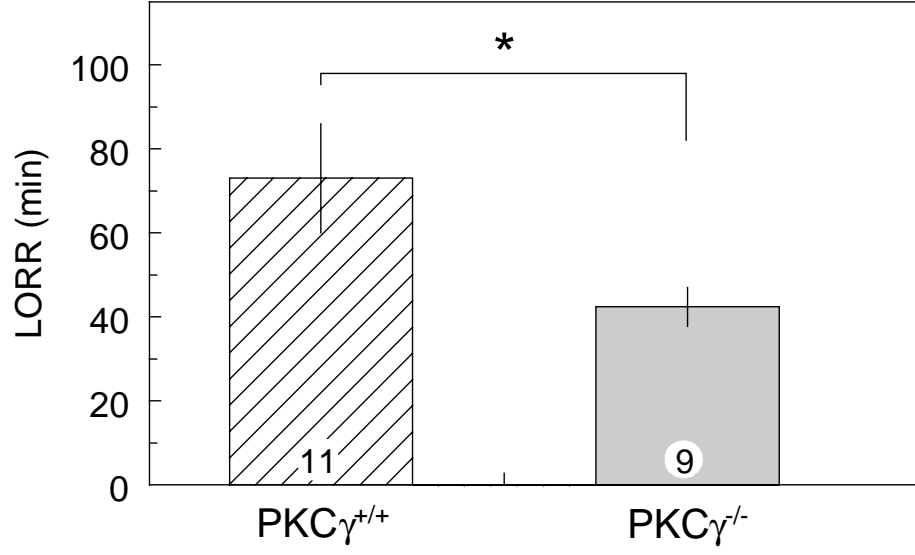
Ethanol effects on holding current, membrane potential and area under the curve of the IPSC response.

Mouse line and genotype (n)	Change in Holding Current (pA)	Membrane Potential (% of baseline)	GABA_A IPSC area (% of baseline)
PKC $\gamma^{+/+}$ (26)	9.62 \pm 2.8*	101 \pm 4.0	130 \pm 8.4*
PKC $\gamma^{-/-}$ (32)	0.44 \pm 5.2	99.0 \pm 2.4	111 \pm 5.03
PKC $\epsilon^{+/+}$ (33)	9.31 \pm 3.2*	97.3 \pm 1.9	108 \pm 2.6
PKC $\epsilon^{-/-}$ (27)	11.8 \pm 3.6*	97.5 \pm 3.1	132 \pm 6.8*

Values are mean \pm s.e.m; * $P < 0.05$ compared with baseline values; (n) is the number of cells tested.

Fig. 1

A



B

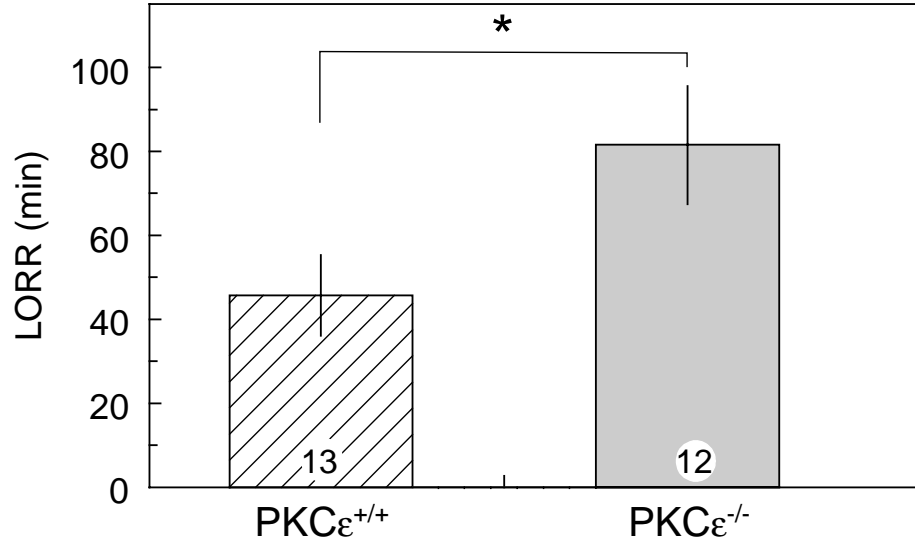


Fig. 2

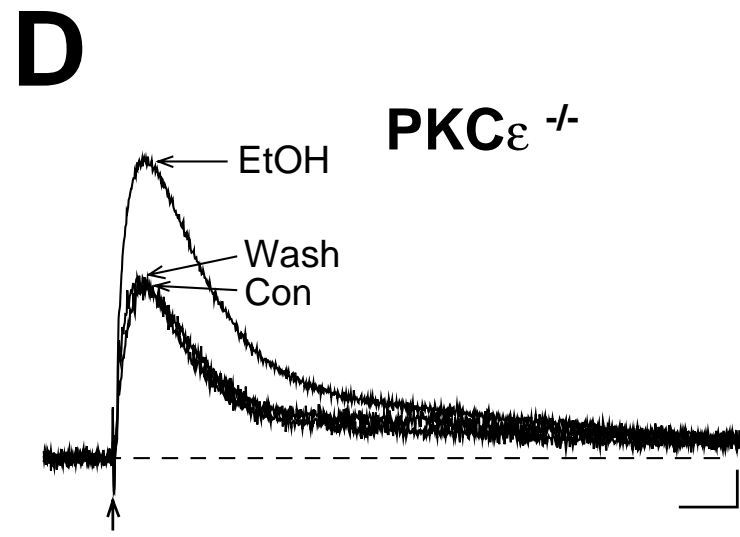
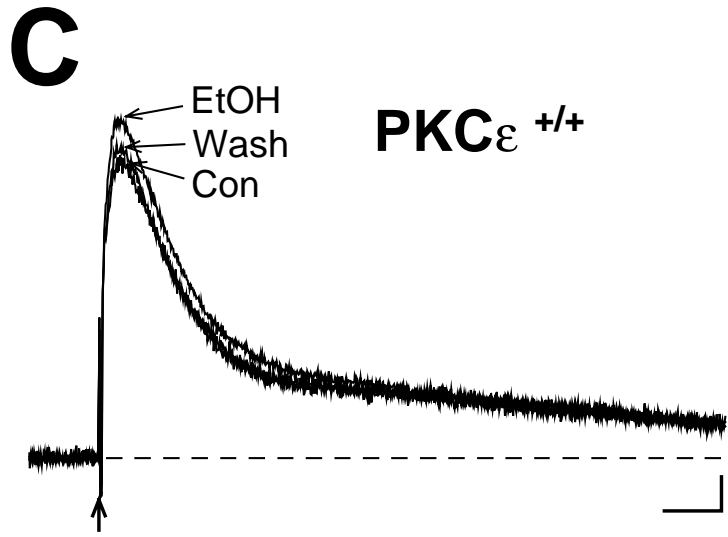
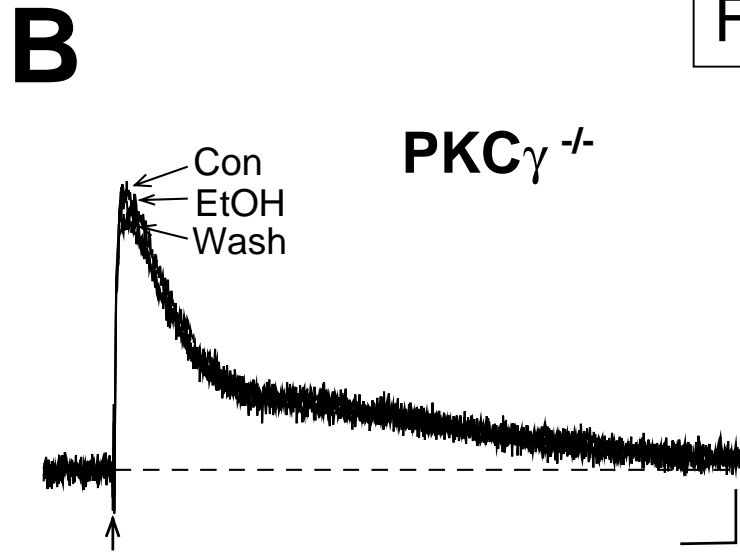
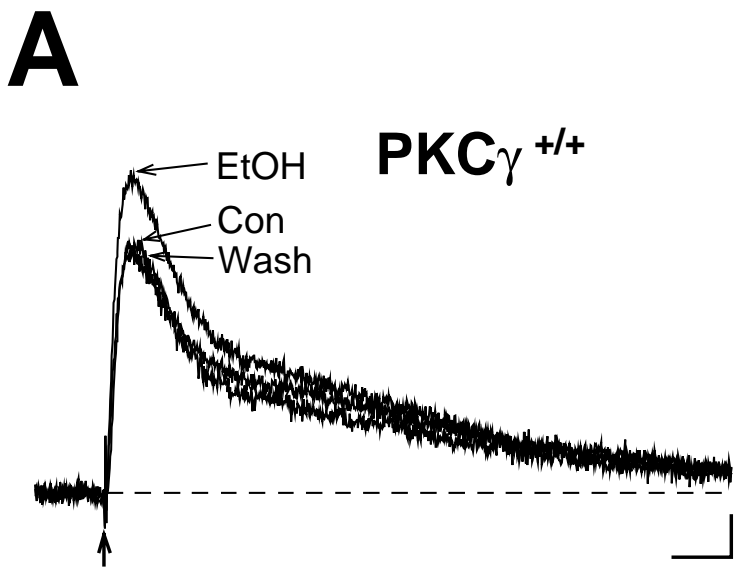


Fig. 3

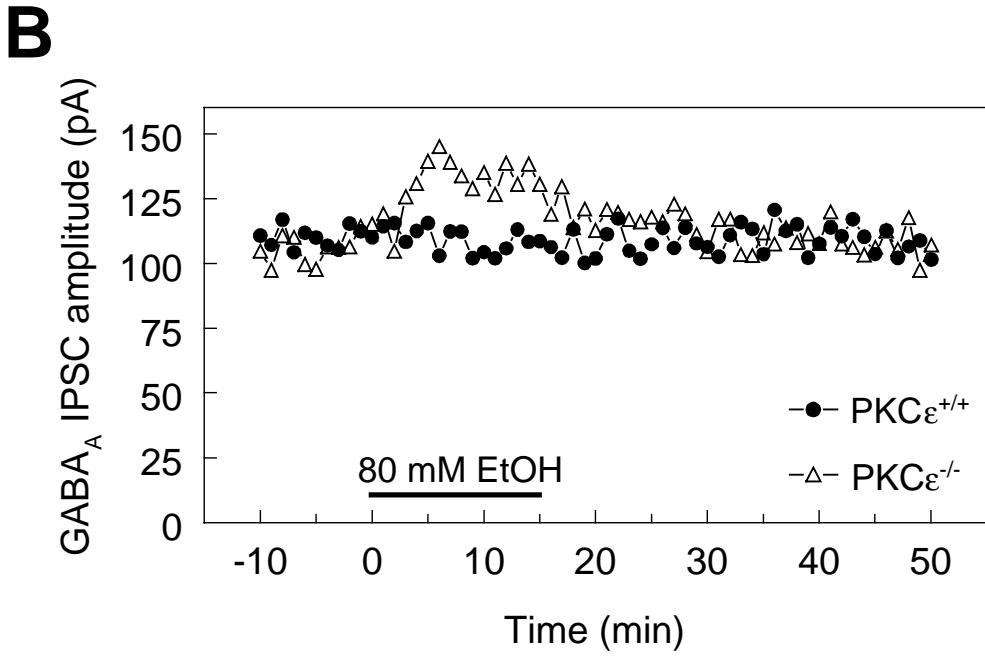
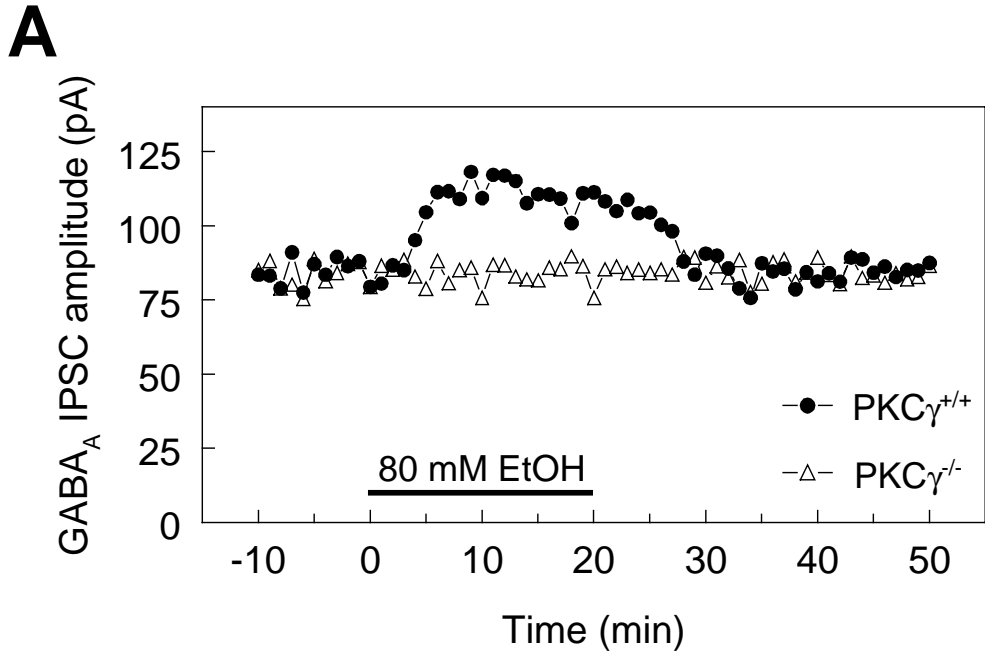


Fig. 4

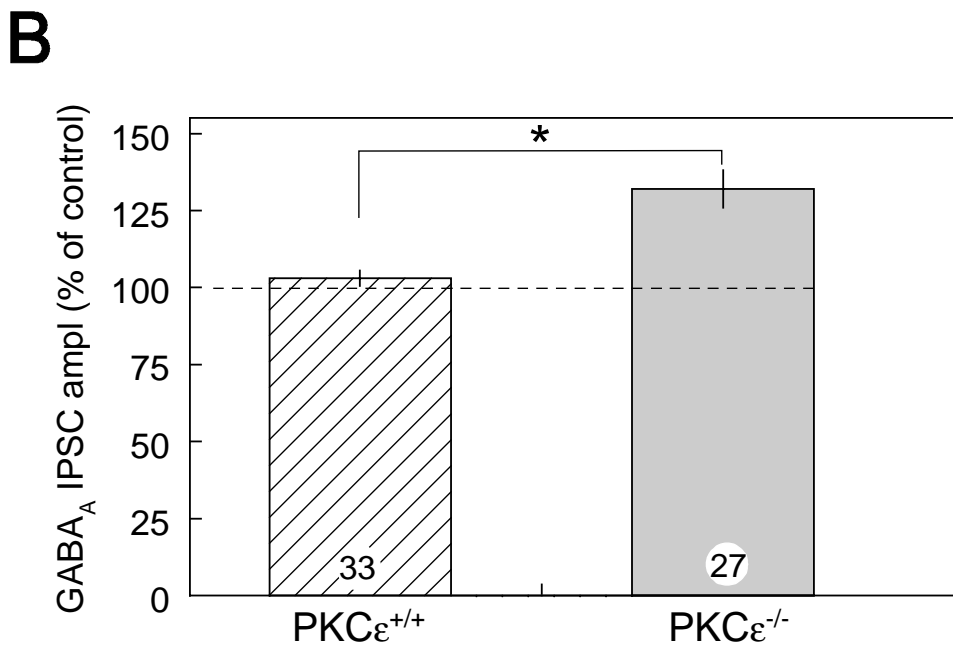
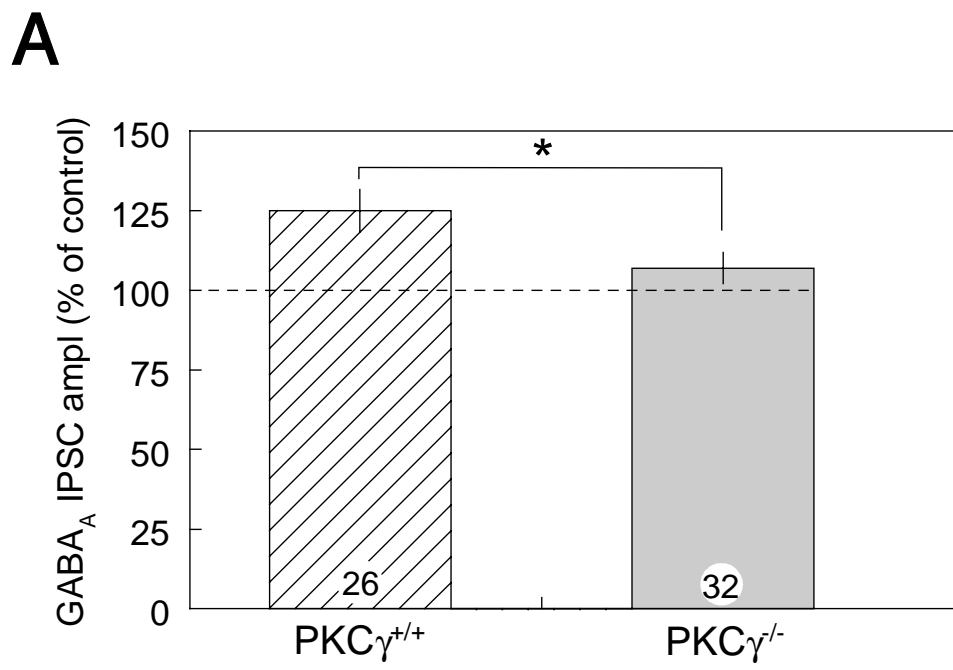


Fig. 5

