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GABA_A Receptor Regulation of Voluntary Ethanol Drinking Requires PKC ϵ

Joyce Besheer, Veronique Lepoutre, Beth Mole, and Clyde W. Hodge*

Bowles Center for Alcohol Studies, Department of Psychiatry, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract

Protein kinase C (PKC) regulates a variety of neural functions, including ion channel activity, neurotransmitter release, receptor desensitization and differentiation. We have shown previously that mice lacking the ϵ -isoform of PKC (PKC ϵ) self-administer 75% less ethanol and exhibit supersensitivity to acute ethanol and allosteric positive modulators of GABA_A receptors when compared with wild-type controls. The purpose of the present study was to examine involvement of PKC ϵ in GABA_A receptor regulation of voluntary ethanol drinking. To address this question, PKC ϵ null-mutant and wild-type control mice were allowed to drink ethanol (10% v/v) vs. water on a two-bottle continuous access protocol. The effects of diazepam (nonselective GABA_A BZ positive modulator), zolpidem (GABA_A α 1 agonist), L-655,708 (BZ-sensitive GABA_A α 5 inverse agonist), and flumazenil (BZ antagonist) were then tested on ethanol drinking. Ethanol intake (grams/kg/day) by wild-type mice decreased significantly after diazepam or zolpidem but increased after L-655,708 administration. Flumazenil antagonized diazepam-induced reductions in ethanol drinking in wild-type mice. However, ethanol intake by PKC ϵ null mice was not altered by any of the GABAergic compounds even though effects were seen on water drinking in these mice. Increased acute sensitivity to ethanol and diazepam, which was previously reported, was confirmed in PKC ϵ null mice. Thus, results of the present study show that PKC ϵ null mice do not respond to doses of GABA_A BZ receptor ligands that regulate ethanol drinking by wild-type control mice. This suggests that PKC ϵ may be required for GABA_A receptor regulation of chronic ethanol drinking.

Keywords

ethanol drinking; PKC; PKC ϵ ; GABA; GABA_A; diazepam; zolpidem; L-655; 708; benzodiazepine

INTRODUCTION

Protein kinase C (PKC) is a family of serine–threo-nine kinases that is divided into three major subsets: conventional (α , β I, β II, and γ), novel (δ , ϵ , Z, and θ), and atypical (λ and ζ), based primarily on structural and functional properties of the kinase regulatory domain (Newton, 2003). In general, PKC regulates a variety of neural functions, including ion channel activity, neurotransmitter release, receptor desensitization and differentiation (Tanaka and Nishizuka, 1994). Evidence suggests that specific isoforms of PKC (PKC γ and PKC ϵ) regulate the biochemical and behavioral effects of ethanol (Harris et al., 1995; Hodge et al., 1999). Mice lacking the PKC ϵ isozyme show greater sensitivity to low and high doses of ethanol (Hodge et al., 1999). Consistent with increased acute sensitivity to ethanol, PKC ϵ null-mutant mice

have been shown to self-administer less ethanol than do wild-type controls under voluntary home-cage (Hodge et al., 1999) and operant self-administration (Olive et al., 2000) conditions. PKC ϵ null mice also exhibit reduced relapse to ethanol self-administration (Olive et al., 2000). These findings are consistent with the hypothesis that increased acute sensitivity to ethanol is negatively correlated with ethanol consumption in animals (Blednov et al., 2003; Thiele et al., 1998) and alcoholism risk in humans (Schuckit, 1994).

Evidence also indicates that ethanol self-administration is regulated by GABA $_A$ receptor activity (Boyle et al., 1993; Shelton and Grant, 2001; Smith et al., 1992; Wegelius et al., 1994), and PKC ϵ can influence GABA $_A$ -mediated actions of ethanol (Hodge et al., 1999). Accordingly, PKC ϵ null-mutant mice show increased behavioral (motor activation and hypnosis) and biochemical (Cl $^-$ flux) sensitivity to allosteric positive modulation of GABA $_A$ receptors (Hodge et al., 1999), suggesting that PKC ϵ regulates GABA $_A$ receptor function (Proctor et al., 2003). Consistent with these results from null mutant mice, PKC ϵ is abundant in brain regions (Saito et al., 1993) where GABA $_A$ receptors are known to influence ethanol self-administration, such as the nucleus accumbens and amygdala (Hodge et al., 1995, 1996; Hyttia and Koob, 1995; Roberts et al., 1996). These data suggest that PKC ϵ -regulated changes in GABA $_A$ receptor sensitivity may regulate ethanol self-administration.

Although it is not yet known which GABA $_A$ receptor subunit(s) are required for PKC modulation of ethanol-associated effects, PKC ϵ has been shown to be colocalized with benzodiazepine (BZ)-sensitive GABA $_A$ receptors (i.e., $\alpha 1$ subunit containing) in the nucleus accumbens and amygdala (Olive and Hodge, 2000). BZ-sensitive GABA $_A$ receptors have been shown to modulate voluntary ethanol drinking (June et al., 1996; Schmitt et al., 2002). It is not known, however, whether ethanol drinking is modulated by interactions between PKC ϵ and BZ-sensitive GABA $_A$ receptors.

Therefore, the purpose of this study was to examine involvement of BZ-sensitive GABA $_A$ receptors in voluntary ethanol drinking by PKC ϵ null-mutant and wild-type control mice. To accomplish this goal, mice were first exposed to ascending concentrations of ethanol (2–18% (v/v) vs. H $_2$ O) and then maintained at 10% ethanol (v/v) vs. H $_2$ O for the duration of the study. Then, the effects of the nonselective GABA $_A$ BZ positive modulator diazepam, the GABA $_A$ $\alpha 1$ agonist zolpidem, the BZ-sensitive GABA $_A$ $\alpha 5$ inverse agonist L-655,708, and the BZ antagonist flumazenil were tested on ethanol drinking. Finally, to confirm involvement of BZ-sensitive GABA $_A$ receptors in modulating ethanol drinking, animals were pretreated with the BZ antagonist flumazenil before diazepam administration and ethanol intake was measured. Given that these animals are bred on a different genetic background when compared with previous studies, we also sought to replicate previously reported differences in ethanol and diazepam acute sensitivity (i.e., Hodge et al., 1999).

METHODS

Animals

Male PKC ϵ wild-type (PKC $\epsilon^{+/+}$) and PKC ϵ null-mutant mice (PKC $\epsilon^{-/-}$) weighing between 25 and 35 g were used in the present study. PKC ϵ null mice were first derived by homologous recombination in J1 embryonic stem cells (Khasar et al., 1999) and have now been backcrossed to each of the inbred mouse strains C57BL/6J and 129SvJae. PKC ϵ heterozygous null mice from each strain were mated to produce male F1 hybrid null and wild-type controls for use in the present study. This breeding scheme is considered to be a rigorous control for genetic background of experimental animals and is in agreement with the recommendations of the Branbury Conference on Genetic Background in Mice (Silva et al., 1997). Mice were housed individually (drinking studies) or in groups of 3–4 (acute response studies) in standard Plexiglas cages with food and water available continuously. The colony room was maintained on a

reverse 12-h light/dark cycle with lights off at 2200 hrs. All procedures were in accordance with the *NIH Guide to Care and Use of Laboratory Animals* and institutional guidelines.

Apparatus

Eight Plexiglas chambers (28 × 28 cm²; Med Associates) were used to measure locomotor activity. Each chamber was equipped with two sets of 16 pulse-modulated infrared photo beams (2.5 cm from the floor) placed on opposite walls to record x-y ambulatory movements. Activity chambers were computer interfaced (Med Associates) for data sampling at 100-ms resolution.

Procedures

Alcohol drinking and testing—Mice (PKCε^{+/+}, *n* = 6; PKCε^{-/-}, *n* = 5) were given access to two 50-ml plastic bottles equipped with ballbearing stoppers to limit spillage. Throughout the duration of the study, mice always had access to 2 bottles, one containing ethanol and the other water. The position of the bottles (left or right side) was alternated daily. Mice were weighed and the volumes were recorded daily ~30 min prior to the onset of the dark cycle. In order to investigate acquisition of ethanol drinking behavior in each group, mice were exposed to ascending ethanol concentrations (2, 4, 8, 10, 14, 18%, v/v) for 4 days at each concentration. After the ethanol dose–response curve was determined, mice were maintained at 10% ethanol (v/v) for the duration of the study.

In order to assess benzodiazepine (BZ)-sensitive GABA_A modulation of ethanol drinking, the initial assessment involved administration of diazepam (0, 10 mg/kg, i.p.). Second, the effects of the BZ-sensitive GABA_A α1 agonist zolpidem were tested (0, 10 mg/kg, i.p.). L-655,708 (0, 1 mg/kg, i.p.) was used to test the effects of the BZ-sensitive GABA_A α5 inverse agonist, and flumazenil (0, 5 mg/kg, i.p.) was used to test the effects of a BZ-antagonist on ethanol drinking. Finally, the ability of flumazenil to antagonize the effects of diazepam on ethanol drinking was tested. Specifically, mice were pretreated with flumazenil (0, 5 mg/kg) 10 min before receiving diazepam (0, 10 mg/kg). For all the tests in this study (except when mentioned), the test compound was administered at the onset of the 24 h cycle with no more than two tests per week (Tuesdays and Thursdays). There was at least 1 week between testing of different compounds.

Ethanol-induced hypnosis—Mice (PKCε^{+/+}, *n* = 4; PKCε^{-/-}, *n* = 3) were injected with a sedative dose of ethanol (3.5 g/kg, i.p.) and intermittently placed on their backs in a v-shaped trough to test for the loss of righting reflex. Loss of righting was defined as the inability of an animal to right itself after being placed on its back within a 30-s interval. Recovery of the righting reflex was determined when the animal righted itself three times within 30 s. Duration was defined as the time interval between loss and recovery of the righting reflex.

Locomotor activity—To test the effects of ethanol (2 g/kg) and diazepam (1.5 mg/kg) on locomotor ability, mice were allowed 1 h to habituate to the activity chambers. After 1 h, mice were removed from the chamber and injected i.p. with ethanol or diazepam and immediately returned to the chamber. For the ethanol test (PKCε^{+/+}, *n* = 12; PKCε^{-/-}, *n* = 10), locomotor activity was monitored for 5 min based on preliminary data showing greatest ethanol-induced effect within the first 5 min, and for the diazepam test (PKCε^{+/+}, *n* = 4; PKCε^{-/-}, *n* = 3), locomotor activity was monitored for 1 h.

Drugs

For acute injection (ethanol sedation and locomotor assessment), ethanol (95%, w/v) was diluted in saline to a concentration of 20% (v/v) and administered i.p. in various volumes to obtain the appropriate dose (gram per kilogram). For alcohol drinking, ethanol (95%, w/v) was diluted in water to the desired concentration. Diazepam, flumazenil and zolpidem (Sigma-

Aldrich, St. Louis, MO) and their vehicles, 1, 0.5, and 0.5% carboxymethylcellulose, respectively, were injected i.p. at a volume of 10 ml/kg. L-655,708 (Tocris, Ellisville, MO) was diluted in a vehicle containing saline (80%) and DMSO (20%) vehicle and injected at a volume of 10 ml/kg.

Data analysis

Duration of the loss of righting reflex and the locomotor assessments were analyzed using *t*-tests. Two-bottle drinking data were analyzed using two-way analysis of variance (ANOVA), with ethanol concentration or pretreatment drug dose as a repeated factor. Tukey's post hoc tests were used to extract significant main effects and interactions. In the absence of a significant interaction, within genotype planned comparisons were used to follow-up a significant main effect of ethanol concentration/drug dose to determine whether drug treatment altered ethanol intake relative to vehicle injection.

RESULTS

Alcohol drinking

Exposure to ascending concentrations of ethanol was not accompanied by a significant difference in genotype, as both the PKC ϵ null mutants and PKC ϵ wild-type mice showed similar ethanol intake (g/kg; Table I). There was, however, a significant main effect of ethanol dose [$F(5,45) = 39.56, P < 0.001$]. In PKC ϵ wild-type mice, greater ethanol intake was observed at all concentrations relative to 2% ethanol, $P_s < 0.006$; in PKC ϵ null-mutant mice, greater ethanol intake was observed at all concentrations (except 8%) relative to 2%, $P_s < 0.02$. The genotype x ethanol concentration interaction was not significant. Ethanol intake is also expressed as milliliters consumed. As with the gram per kilogram analysis, there was a significant main effect of ethanol dose [$F(5,45) = 3.37, P = 0.01$], which was driven by a downward trend in milliliters consumed by the PKC ϵ null-mutant mice and a significant decrease in the wild-type mice at the 14% ethanol concentration, $P = 0.047$. There was no significant difference in genotype and the genotype x ethanol concentration interaction was not significant. Water consumption was not altered by exposure to the ascending ethanol concentrations, and PKC ϵ null-mutant and PKC ϵ wild-type mice did not differ in overall water consumption (Table I).

Modulation of ethanol drinking by diazepam (10 mg/kg), zolpidem (10 mg/kg), flumazenil (5 mg/kg), and L-655,708 (1 mg/kg)

Diazepam selectively reduced ethanol intake (g/kg) as shown by a significant genotype x diazepam treatment interaction [$F(1,9) = 6.86, P = 0.03$; Fig. 1A]. Specifically, diazepam treatment significantly reduced ethanol intake in the PKC ϵ wild-type mice, $P = 0.006$, with no effect of ethanol intake in the null-mutant mice. PKC $\epsilon^{+/+}$ mice consumed significantly greater ethanol relative to the null-mutant mice, $P = 0.03$, after vehicle treatment; diazepam treatment eliminated this difference. The PKC ϵ wild-type and null-mutant mice did not differ on water intake (Table II). However, diazepam (10 mg/kg) treatment increased water intake [$F(1,9) = 7.93, P = 0.02$; Table II], with greater water intake after diazepam treatment in the PKC ϵ wild-type mice, $P = 0.045$. The genotype x diazepam treatment interaction was not significant. Overall, there was no change in total fluid consumption (ethanol + water, milliliters), which indicates that the diazepam-induced reduction in ethanol intake was accompanied by a compensatory increase in water intake.

Zolpidem (10 mg/kg) treatment significantly reduced ethanol intake [$F(1,9) = 7.10, P = 0.03$; Fig. 1B], with a significant zolpidem-induced reduction in PKC ϵ wild-type mice, $P = 0.049$. No change was observed in the PKC ϵ null-mutant mice. There was no significant main effect of genotype or interaction. Zolpidem also significantly reduced water intake [$F(1,9) = 56.36, P < 0.001$; Table II] in both PKC ϵ wild-type mice and null mutant mice, $P_s < 0.03$. Overall,

zolpidem decreased total fluid consumption [ethanol + water, milliliters; $F(1,9) = 54.99$, $P < 0.001$], in both PKC ϵ wild-type and null mutant mice, $P_s = 0.002$, which suggests a nonspecific effect on drinking behavior.

The effects of L-655,708 (1 mg/kg) treatment on ethanol intake are shown in Figure 1C. The ethanol and water intake data from one PKC ϵ wild-type mouse are not included in this analysis because of a leak in the ethanol bottle. There was a significant main effect of genotype [$F(1,9) = 24.14$, $P < 0.001$], and a significant interaction [$F(1,8) = 5.76$, $P = 0.04$]. L-655,708 significantly increased ethanol intake in PKC ϵ wild-type mice, $P = 0.02$, with no effect on ethanol intake in PKC ϵ null-mutant mice. PKC ϵ wild-type mice consumed significantly greater ethanol relative to the null-mutant mice, $P = 0.04$, after vehicle treatment and L-655,708 treatment, $P < 0.001$. Water intake was also altered by L-655,708 treatment (Table II). A significant main effect of genotype [$F(1,9) = 8.80$, $P = 0.02$] and a significant dose x genotype interaction [$F(1,8) = 8.19$, $P = 0.02$] were found. L-655,708 did not affect water intake in the PKC ϵ null-mutant mice; however, a significant decrease was observed in the PKC ϵ wild-type mice, $P = 0.02$, which likely represents a compensatory decrease in fluid intake, corresponding to the increase in ethanol intake. Water intake between the genotypes did not differ after vehicle injection; however, PKC ϵ wild-type mice did show lower levels of water intake relative to the null-mutant mice after 1 mg L-655,708/kg, $P < 0.001$. Overall, there was no change in total fluid consumption (ethanol + water, milliliters), which indicates that the increase in ethanol intake produced by L-655,708 was accompanied by a compensatory decrease in water intake.

Flumazenil (5 mg/kg) treatment did not affect ethanol consumption (Fig. 1D). A significant main effect of genotype was observed [$F(1,9) = 15.73$, $P = 0.003$] with the PKC ϵ wild-type mice exhibiting higher levels of ethanol intake than the null-mutant mice. The interaction was not significant. Flumazenil treatment significantly increased water intake (Table II), as evident by a significant main effect of drug treatment [$F(1,9) = 7.61$, $P = 0.02$], with an increase in water intake in the PKC ϵ null-mutant mice, $P = 0.03$. Overall, there was no change in total fluid (ethanol + water, milliliters) consumed.

Flumazenil blockade of diazepam-induced drinking

Pretreatment with flumazenil (5 mg/kg) significantly altered the pattern of drinking induced by diazepam (10 mg/kg), as shown in Figure 2. A significant main effect of drug [$F(2,18) = 3.98$, $P = 0.04$] and a significant genotype x drug interaction [$F(2,18) = 10.31$, $P = 0.001$] were observed. In the PKC ϵ wild-type mice, diazepam treatment significantly reduced ethanol intake, $P < 0.001$, as previously observed. Further, flumazenil pretreatment prevented the diazepam-induced reduction, as ethanol intake was similar to vehicle alone levels, and significantly greater than diazepam alone levels, $P = 0.004$. In the PKC ϵ null-mutant mice, diazepam did not significantly alter ethanol intake, consistent with our previous findings (Fig. 1A), and flumazenil pretreatment had no effect on the pattern of diazepam-induced drinking. Further, with vehicle alone, ethanol intake was significantly greater in PKC ϵ wild-type than in null-mutant mice, $P = 0.007$. This difference was prevented by diazepam treatment and flumazenil pretreatment. Water intake was not influenced by drug treatment or genotype (Table III). A significant interaction was observed [$F(2,18) = 3.70$, $P = 0.045$]; however, no significant differences were detected by the Tukey post hoc tests. Overall, flumazenil blocked the effects of diazepam on both ethanol and water intake, resulting in no change in total fluid (ethanol + water, milliliters) consumed.

Ethanol sedation and locomotor activity

As shown in Figure 3A, ethanol (3.5 g/kg) significantly increased the duration of the loss of righting reflex in the PKC ϵ null-mutant mice, $P = 0.02$. The tests of ethanol and diazepam-induced locomotor activity are illustrated in Figure 3B (left and right panel respectively).

PKC ϵ null-mutant mice showed greater locomotor response to an acute administration of ethanol (2 g/kg), $P = 0.03$, and to acute administration of diazepam (1.5 mg/kg), $P = 0.006$, than did PKC ϵ wild-type mice. These findings confirm the results of previous work showing greater sensitivity to ethanol-induced sedation and acute administration of ethanol and diazepam in PKC ϵ null-mutant mice (Hodge et al., 1999).

DISCUSSION

The primary goal of this study was to examine potential interactions between PKC ϵ and GABA $_A$ receptors in regulation of alcohol self-administration. Substantial evidence indicates that GABA $_A$ receptors modulate alcohol self-administration (Hodge et al., 1995, 1996; June et al., 2003; McKay et al., 2004; Rassnick et al., 1993; Roberts et al., 1996). Alcohol exposure and withdrawal produces adaptations in GABA $_A$ systems (Crews et al., 1996; Kumar et al., 2003; Morrow et al., 1988), which may increase subsequent alcohol self-administration behavior (Roberts et al., 1996) and function through a PKC mechanism (Kumar et al., 2002). Moreover, PKC ϵ null mice are supersensitive to behavioral and biochemical effects of acute alcohol and GABA $_A$ positive modulators (Hodge et al., 1999). This supersensitivity is associated with reduced alcohol self-administration behavior and ethanol withdrawal seizure severity (Hodge et al., 1999; Olive et al., 2000, 2001). We have shown that PKC ϵ is colocalized with BZ-sensitive GABA $_A$ $\alpha 1$ containing receptors in the nucleus accumbens (Olive and Hodge, 2000). This suggests that PKC ϵ may modulate the GABA $_A$ -receptor-mediated properties of ethanol self-administration.

In the present work, ethanol consumption in PKC ϵ null-mutant and wild-type mice did not differ during exposure to ascending ethanol concentrations (2–18%, (v/v) vs. H $_2$ O). However, a significant difference in ethanol intake emerged after completion of the ascending concentration curve with less ethanol (10%, v/v) consumption in PKC ϵ null-mutant mice than in wild-type controls, which agrees with previously published results (Hodge et al., 1999). It is unclear why the PKC ϵ null mutation did not influence ethanol intake during the initial phase of ethanol exposure (i.e., ascending concentrations), which is in contrast with published work showing decreased ethanol intake in PKC ϵ null-mutant mice during exposure to a similar ethanol concentration curve (Hodge et al., 1999). One possible explanation for this inconsistency is that the mice used in previous work were F2–F4 hybrid C57BL/6J \times 129 SvJae mice (from initial chimeras) (Khasar et al., 1999) whereas the mice used in the present study were F1 hybrid congenic C57BL/6J \times 129 SvJae mice. This suggests that genetic background or Sv129 flanking genes (Crawley et al., 1997; Gerlai, 2001) may contribute to the apparent involvement of PKC ϵ in regulation of initial ethanol intake. However, the previously reported phenotypic difference emerged and was maintained when mice were exposed to ethanol (10%, v/v) throughout the extended baseline and drug testing phases of the experiment. Thus, it appears that PKC ϵ regulates the maintenance of voluntary ethanol drinking in a manner that may not depend on genetic background.

Results of the present study show that administration of the nonselective BZ positive modulator diazepam (10 mg/kg) or the GABA $_A$ $\alpha 1$ receptor agonist zolpidem (10 mg/kg) decreased voluntary ethanol intake in wild-type mice, but did not affect ethanol drinking in the PKC ϵ null-mutant mice. Importantly, PKC ϵ null mice showed significant changes in water drinking, indicating that drug doses were behaviorally active and that drinking patterns can be modulated by GABA $_A$ ergics in these mice. The diazepam-induced reduction of ethanol intake was completely blocked by pretreatment with the BZ antagonist flumazenil, confirming involvement of GABA $_A$ BZ receptors in wild-type mice. Previous work has found that treatment with BZs increases (Ingman et al., 2004; Soderpalm and Hansen, 1998; Wegelius et al., 1994) and decreases (Hedlund and Wahlstrom, 1998; Petry, 1995; Samson and Grant, 1985) ethanol intake. The effects of the compounds can vary by different factors such as BZ

dose, self-administration protocol (i.e., continuous access, limited access—for a review, see Chester and Cunningham, 2002), and ethanol self-administration dose. The findings of the present work are consistent with diazepam (20 mg/kg)-induced decreases in ethanol intake using a continuous access two-bottle voluntary drinking procedure in rats (Hedlund and Wahlstrom, 1998). Flumazenil, the BZ receptor antagonist, has been shown to reverse reductions in drinking induced by GABA_A/BZ partial inverse agonists (June et al., 1992; McBride et al., 1988); however, to our knowledge the effects of a BZ receptor antagonist on diazepam-induced reductions in ethanol intake have not been evaluated. Pretreatment with the GABA_A α 5 partial inverse agonist L-655,708 increased ethanol intake in the wild-type mice, but was without effect on ethanol consumption in the PKC ϵ null-mutant mice. Although L-655,708 has not been previously tested in alcohol self-administration studies, this result is consistent with its anxiogenic effects (Navarro et al., 2002) and general association between anxiety and alcohol drinking (Pandey, 2003).

Overall, these results appear inconsistent with previously published results. That is, given that PKC ϵ null-mutant mice show greater sensitivity to allosteric positive modulation of GABA_A receptors (Hodge et al., 1999), one would predict greater sensitivity to the modulation of voluntary ethanol drinking by BZ-sensitive GABA_A receptors. However, as discussed earlier, the BZ compounds tested were without effect on ethanol drinking in the PKC ϵ null-mutant mice. To confirm previously reported differences in acute sensitivity to ethanol and diazepam, PKC ϵ null-mutant mice were exposed to acute administration of ethanol and diazepam. Consistent with previous findings, PKC ϵ null-mutant mice showed greater sensitivity to a hypnotic dose of ethanol (3.5 g/kg) as indicated by a significant increase in the duration of the loss of righting reflex relative to PKC ϵ wild-type mice. Also consistent with previous work, PKC ϵ null-mutant mice showed greater locomotor activity in response to 2 g ethanol/kg and 1.5 mg diazepam/kg (Hodge et al., 1999) than did PKC ϵ wild-type mice. These data demonstrate that previously published phenotypes remain intact in these mice and that changes in GABA_A or ethanol sensitivity do not account for the lack of response to GABAergic drugs by the PKC ϵ null mice.

Thus, it is important to consider why PKC ϵ null mice are more sensitive to the acute effects of ethanol and GABA_A receptor positive modulators but show an apparent lack of sensitivity to modulation of ethanol self-administration by this receptor system. First, in this study mice chronically self-administered ethanol for an extended period, which may have induced adaptive changes in wild-type mice that were absent in PKC ϵ mutants. For example, *in vitro* evidence shows that chronic ethanol increases the abundance of PKC ϵ (Messing et al., 1991), which may decrease behavioral and biochemical sensitivity to ethanol and/or GABA_A receptor compounds (Hodge et al., 1999, 2002). PKC ϵ is colocalized with BZ-sensitive GABA_A α 1-containing receptors in a variety of limbic brain regions (Olive and Hodge, 2000) and chronic ethanol increases receptor binding of compounds that target this subtype of the GABA_A receptor (Devaud et al., 1995; Mhatre et al., 1988). Thus, upregulation of PKC ϵ by chronic ethanol could contribute to altered sensitivity to BZs or ethanol in wild-type mice, which would be absent in PKC ϵ null-mutant mice. Second, since PKC ϵ null mice show heightened GABA_A receptor responses to acute ethanol (Hodge et al., 1999; Proctor et al., 2003), chronic ethanol might lead to a downregulation of GABA_A receptor function or expression in the null mice, which could result in decreased sensitivity (e.g., tolerance) to ethanol or GABAergic compounds.

There are several issues related to the present study that merit discussion. First, the effects of the GABA-ergic compounds were tested using a repeated measures design, which has the potential to confound order of testing with individual drug effects. For example, chronic ethanol exposure or repeated drug testing may have altered response to the GABAergic compounds. However, the effects of diazepam were constant across two separate tests, indicating no change

in response after repeated testing. Second, although acute ethanol (4 g/kg, i.p.) clearance does not differ between PKC ϵ null-mutant and wild-type mice (4 g/kg, i.p.; Hodge et al., 1999), measurement of blood ethanol concentrations in the present work would have allowed us to determine whether PKC ϵ regulates ethanol metabolism after chronic exposure. However, baseline ethanol intake remained stable throughout the experiment in both PKC ϵ null mice and wild-type controls, suggesting that ethanol metabolism was not changed. Third, while acute response to ethanol and diazepam confirmed previously published phenotypes (e.g., Hodge et al., 1999), conducting the same tests after ethanol self-administration would have allowed a direct assessment of adaptive changes in ethanol or GABAergic sensitivity. More research is needed to address these issues and potential changes in PKC ϵ regulation of GABA $_A$ receptor function induced by chronic ethanol self-administration.

In conclusion, results of this study show that PKC ϵ null mice do not respond to doses of GABA $_A$ BZ receptor ligands that decrease (diazepam and zolpidem), or increase (L-655,708), ethanol drinking in wild-type mice. This lack of response by the PKC ϵ null mice occurs in spite of increased sensitivity to acute ethanol and diazepam. These findings suggest that GABA $_A$ receptor regulation of ethanol self-administration requires PKC ϵ .

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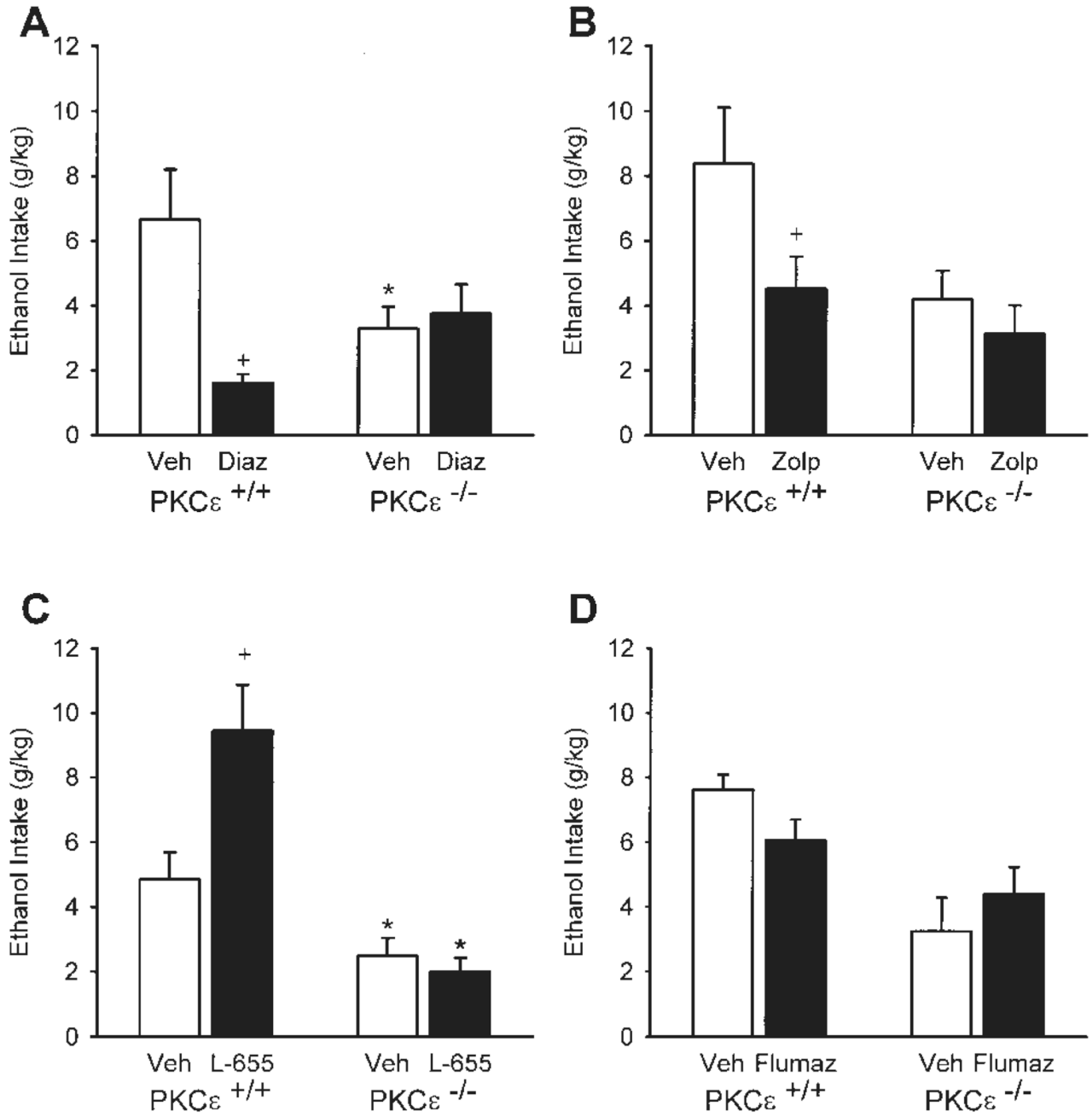


Fig. 1. Ethanol intake (\pm SEM; in gram per kilogram) by PKC ϵ wild-type ($^{+/+}$) and null-mutant ($^{-/-}$) mice after administration of 10 mg diazepam/kg (Diaz, **A**), 10 mg zolpidem/kg (Zolp, **B**), 1 mg L-655,708/kg (L-655, **C**), and 5 mg flumazenil/kg (Flumaz, **D**). * Denotes significant difference between genotypes at the respective dose (Tukey, $P < 0.05$). + Denotes significant difference from vehicle within genotype. For all compounds, $n = 6$ and 5 for PKC ϵ $^{+/+}$ and PKC ϵ $^{-/-}$ respectively, except for L-655,708, wherein $n = 5$ for both PKC ϵ $^{+/+}$ and PKC ϵ $^{-/-}$.

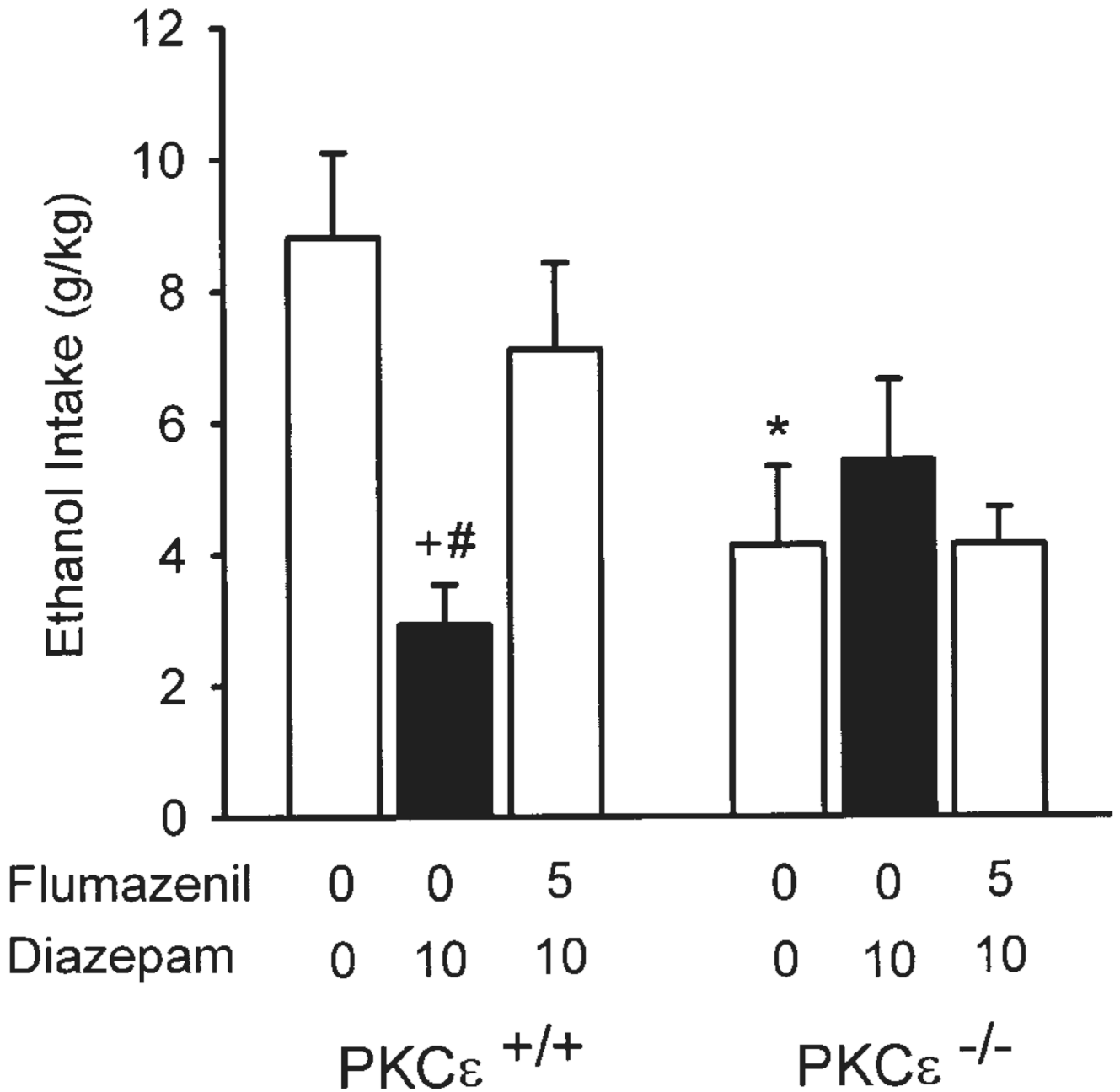
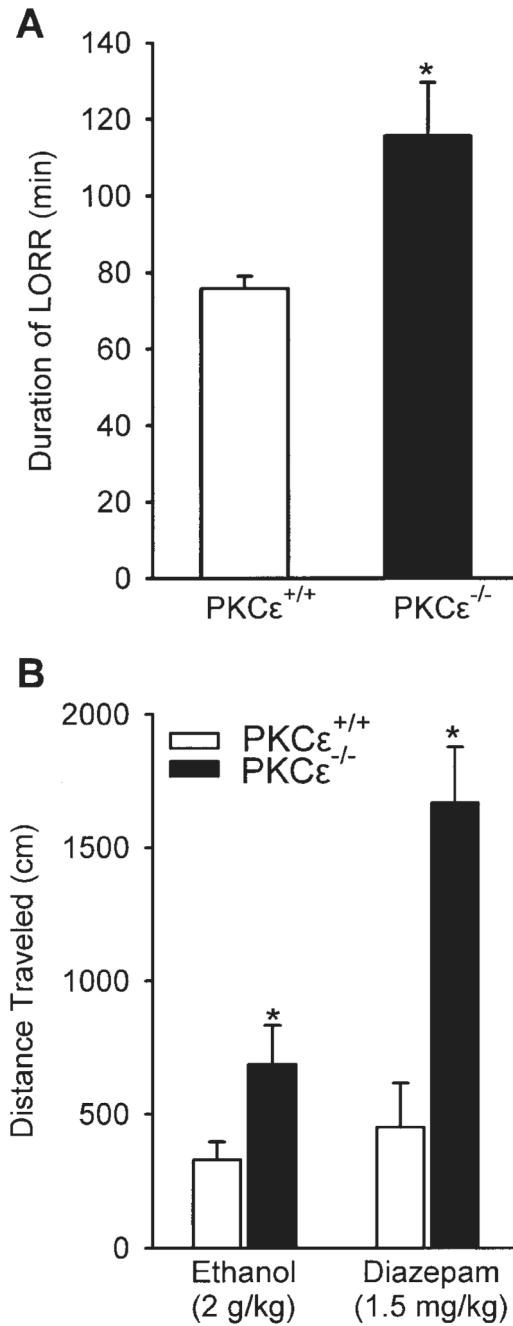


Fig. 2. Ethanol intake (\pm SEM; gram per kilogram) in PKC ϵ wild-type (+/+; $n = 6$) and null-mutant (-/-; $n = 5$) mice after pre-treatment with flumazenil, followed by diazepam 10 min later. * Denotes significant difference between genotypes at the respective dose (Tukey, $P < 0.05$). + Denotes significant difference from vehicle (0 + 0) within genotype (Tukey, $P < 0.05$). # Denotes significant difference from 5 mg flumazenil/kg + 10 mg diazepam/kg treatment (Tukey, $P < 0.05$).

**Fig. 3.**

A. Duration (min) \pm SEM of the loss of righting reflex induced by 3.5 g ethanol/kg in PKC ϵ wild-type (+/+; $n = 4$) and null-mutant (-/-; $n = 3$) mice. **B.** Distance traveled (cm) \pm SEM by PKC ϵ wild-type (+/+) and null-mutant (-/-) mice during a 5-min ethanol test (2 g/kg, i.p.; PKC ϵ ^{+/+}, $n = 12$; PKC ϵ ^{-/-}, $n = 10$), and a 1-h diazepam test (1.5 mg/kg, i.p.; PKC ϵ ^{+/+}, $n = 4$; PKC ϵ ^{-/-}, $n = 3$). * Denotes significant difference ($P < 0.05$).

TABLE I

Ethanol (g/kg) and water intake (ml) at different ethanol concentrations

	Ethanol concentration (% v/v)					
	2	4	8	10	14	18
Ethanol intake (g/kg)						
PKCε ^{+/+}	2.12 ± 0.21	3.73 ± 0.18*	6.70 ± 0.95*	10.71 ± 1.60*	9.35 ± 1.61*	18.14 ± 2.53*
PKCε ^{-/-}	1.81 ± 0.21	3.66 ± 0.66*	6.38 ± 1.92	7.88 ± 1.13*	8.42 ± 0.75*	14.15 ± 1.76*
Ethanol intake (ml)						
PKCε ^{+/+}	3.79 ± 0.33	3.42 ± 0.19	3.06 ± 0.45	3.90 ± 0.60	2.46 ± 0.44	3.08 ± 0.43
PKCε ^{-/-}	3.30 ± 0.32	3.30 ± 0.52	2.90 ± 0.83	2.90 ± 0.38	2.25 ± 0.21	2.72 ± 0.22
Water intake (ml)						
PKCε ^{+/+}	3.60 ± 0.35	3.77 ± 0.27	3.88 ± 0.61	2.67 ± 0.48	3.96 ± 0.58	4.38 ± 0.57
PKCε ^{-/-}	4.03 ± 0.26	3.73 ± 0.29	3.68 ± 0.67	4.23 ± 0.74	4.40 ± 0.62	4.85 ± 0.65

Values given are mean ± SEM.

PKCε^{+/+}, *n* = 6; PKCε^{-/-}, *n* = 5.* *P* < 0.05 relative to 2% within genotype.

TABLE II

Water intake for BZ-tests

	Water intake (ml)	
	Vehicle	Diazepam (10 mg/kg)
PKCε ^{+/+} (n = 6)	3.67 ± 0.77	6.58 ± 0.40*
PKCε ^{-/-} (n = 5)	4.70 ± 0.41	5.40 ± 0.58
	Zolpidem (10 mg/kg)	
	Vehicle	Zolpidem (10 mg/kg)
PKCε ^{+/+} (n = 6)	4.00 ± 0.22	1.92 ± 0.30*
PKCε ^{-/-} (n = 5)	3.80 ± 0.34	2.20 ± 0.54*
	L-655,708 (1 mg/kg)	
	Vehicle	L-655,708 (1 mg/kg)
PKCε ^{+/+} (n = 5)	4.90 ± 0.68	2.83 ± 0.60*
PKCε ^{-/-} (n = 5)	5.30 ± 0.44	6.00 ± 0.35
	Flumazenil (5 mg/kg)	
	Vehicle	Flumazenil (5 mg/kg)
PKCε ^{+/+} (n = 6)	3.58 ± 0.91	4.33 ± 0.68
PKCε ^{-/-} (n = 5)	3.10 ± 0.70	4.10 ± 0.51*

Values given are mean ± SEM.

* $P < 0.05$ relative to vehicle within genotype.

TABLE III

Water intake for flumazenil modulation of diazepam-induced drinking

	Water intake (ml)		
	Veh + Veh	Veh + Diaz	Flu + Diaz
PKC $\epsilon^{+/+}$ ($n = 6$)	3.08 \pm 0.54	4.50 \pm 0.37	3.75 \pm 0.36
PKC $\epsilon^{-/-}$ ($n = 5$)	4.00 \pm 0.91	2.90 \pm 0.48	3.00 \pm 1.19

Veh, vehicle; Diaz, 10 mg diazepam/kg; Flu, 5 mg flumazenil/kg.

Values given are mean \pm SEM.