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Mutant mice lacking the γ isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of γ-aminobutyrate type A receptors

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ABSTRACT **Calcium/phospholipid-dependent protein kinase (protein kinase C, PKC) has been suggested to play a role in the sensitivity of γ-aminobutyrate type A ($GABA_A$) receptors to ethanol. We tested a line of null mutant mice that lacks the γ isoform of PKC (PKCγ) to determine the role of this brain-specific isoenzyme in ethanol sensitivity. We found that the mutation reduced the amount of PKCγ immunoreactivity in cerebellum to undetectable levels without altering the levels of the α, β_I, or β_{II} isoforms of PKC. The mutant mice display reduced sensitivity to the effects of ethanol on loss of righting reflex and hypothermia but show normal responses to flunitrazepam or pentobarbital. Likewise, $GABA_A$ receptor function of isolated brain membranes showed that the mutation abolished the action of ethanol but did not alter actions of flunitrazepam or pentobarbital. These studies show the unique interactions of ethanol with $GABA_A$ receptors and suggest protein kinase isoenzymes as possible determinants of genetic differences in response to ethanol.**

Despite their obvious importance, the mechanisms of action of ethanol remain controversial. During the past few years, attention has shifted from nonspecific membrane perturbation to actions on specific brain proteins. Initial biochemical and genetic studies suggested the γ-aminobutyrate type A ($GABA_A$) receptor as a site for alcohol actions (1–4). Subsequent molecular cloning identified the $GABA_A$ receptor as a member of the ligand-gated superfamily of ion channels, leading to experiments showing that all members of this superfamily are sensitive to ethanol (5, 6). However, the mechanism(s) responsible for actions of ethanol on ligand-gated ion channels remains to be elucidated. For the $GABA_A$ receptor, expression of specific subunits in *Xenopus* oocytes combined with mutagenesis of a putative protein kinase C (PKC) phosphorylation site has suggested that subunit phosphorylation is required for ethanol enhancement of GABA action (7, 8). However, it is not known whether results from the oocyte expression system can be generalized to mammalian brain. Although it is clear that not all brain $GABA_A$ receptors are sensitive to ethanol (9–11), the role of PKC in determining ethanol sensitivity of these receptors *in vivo* remains to be elucidated. The $GABA_A$ receptor is also the site of action of other drugs with sedative, anxiolytic, and inebriating actions, such as benzodiazepines and barbiturates. It is possible that enhancement of GABA function by these drugs is affected by PKC activity (12), although the phosphorylation site in the γ2L subunit appears to be important only for ethanol potentiation (7).

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The role of PKC in the function of $GABA_A$ receptors is also controversial. Activation of PKC by phorbol esters markedly inhibits function of $GABA_A$ receptors expressed in oocytes (13–15) but has less effect on native $GABA_A$ receptors of brain membranes (14, 16). In contrast, the function of recombinant $GABA_A$ receptors expressed in fibroblasts is enhanced by catalytically active PKC (17).

Thus, the questions of whether $GABA_A$ receptor function is modulated by PKC *in vivo* and whether actions of ethanol or other drugs on this receptor require PKC activity remain unresolved. Our approach to these problems was to use null mutant mice lacking a neural-specific isoform of PKC (PKCγ) for measurements of drug sensitivity using *in vivo* behavioral assays and *in vitro* ion flux assays of $GABA_A$ receptor function. PKCγ was deleted, or "knocked out," by gene-targeted homologous recombination (18, 19). This isoform of PKC was chosen because it is found only in the central nervous system, where it is found in many neurons but not in other cells (20–23). In the cerebellum, PKCγ is found only in Purkinje cells, where it is postsynaptically localized in dendrites (21–23). This is potentially of particular importance for the actions of ethanol, as the $GABA_A$ receptors of Purkinje cells appear to be important for some behavioral and electrophysiological actions of ethanol (24, 25). Furthermore, PKCγ is not present at birth, but expression is detectable by 7 days of age and reaches adult levels by 14–28 days of age (23). Thus, deletion of this isoform is unlikely to alter early development. Effects on brain development are a significant concern with mutations of genes expressed early in life, but these problems are obviated with these mice because of the late expression of PKCγ. These null mutant mice display modest impairments in tests of learning and memory and slight ataxia but are otherwise normal in appearance (18). Synaptic transmission is normal in hippocampus, but long-term potentiation is impaired (19).

MATERIALS AND METHODS

Mice. Null mutant mice were derived as described (18, 19). Mice were produced by mating heterozygotes, allowing production of the three genotypes from the same parents. Animals were scored as wild-type or mutants either by Southern blot analysis using DNA extracted from a small tail sample (18, 19) or by polymerase chain reaction (PCR) methods. For PCR, DNA was amplified with primers for the neomycin gene insert (26) and for the PKCγ gene. PKCγ primers were 5′-GCTCCGACGAACTCTATGCCA-3′ and 3′-GTGGAGTGAAGCTGCGTGAGA-5′. Reactions were performed with the Invitrogen Optimizer kit with the buffer N system.

Abbreviations: GABA, γ-aminobutyrate; PKC, protein kinase C.
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Drug Administration. Male and female mice of each genotype were tested at 60–90 days of age. Animals were placed in the testing room overnight prior to weighing and testing. All drugs were administered by an intraperitoneal (i.p.) injection. Ethanol and pentobarbital were dissolved in 0.9% NaCl, and flunitrazepam in 0.5% Tween 80. Ethanol (20%, wt/vol) was administered at 0.02 ml/g of body mass; the other drugs were administered at 0.01 ml/g.

Body Temperature. Body temperature was monitored with a Thermalert TH-5 thermometer. Basal body temperatures were recorded 5 min before and 30 min after an injection of ethanol or pentobarbital. Preliminary experiments indicated that maximal response was observed by 30 min. Response to flunitrazepam was recorded at 60 min because the time course for production of maximal body temperature depression induced by flunitrazepam was longer. The difference between pre- and postinjection body temperature was used to determine the differential effects of ethanol between wild-type and mutants.

Duration of Loss of Righting Response. The procedures for measuring the duration of loss of righting response were identical to those used by Marley *et al.* (27). Mice were administered an i.p. injection of ethanol or pentobarbital. When animals became ataxic, they were placed in a V-shaped plastic trough and the time was recorded. Animals were judged to have regained the righting response when they could right themselves three times within 30 sec. The duration of time between the loss and regaining of response was rounded to the nearest minute and used to compare the differential effects of ethanol between the two genotypes.

Blood Ethanol Concentration. To determine the rate of ethanol metabolism, mutants and wild-type mice were administered an i.p. dose of 20% (wt/vol) ethanol, 3.5 mg/g. Ten-microliter samples of blood were collected in heparanized capillary tubes from the retroorbital sinus and immediately added to 200 μl of ice-cold 0.55 M perchloric acid. Samples were collected at 10, 30, 60, 90, 150, 180, and 225 min after injection. At the end of collection all samples were centrifuged for 10 min at 1500 × *g* at 4°C and neutralized to pH 5 by the addition of 200 μl of 0.6 M KOH containing acetic acid. Samples were recentrifuged and the supernatants were used for assay of ethanol concentration by an alcohol dehydrogenase enzymatic spectrophotometric method (28). Ethanol concentrations were determined from a standard curve prepared daily. The assay is useful for determining blood ethanol concentrations of 5–800 mg/dl (1–175 mM). The rate of ethanol clearance was determined between 30 and 225 min by linear regression analysis.

Immunoblots. Mice were killed by cervical dislocation, and cerebella were rapidly removed on ice. The tissue was homogenized in 0.32 M sucrose. The homogenate was spun at 900 × *g* for 10 min at 10°C. The supernatant was then spun at 56,000 × *g* in a Beckman L3-50 ultracentrifuge for 30 min at 10°C. The supernatant was saved as the cytosolic fraction and the pellet was resuspended in 0.32 M sucrose as the particulate fraction. Protein was determined by the method of Lowry *et al.* (29). The linear range was determined for each isotype examined, and equivalent amounts of protein (depending on PKC isotype) from wild type and mutants were electrophoresed in SDS/10% polyacrylamide gels. The α and γ isotypes were linear in the range 10–50 μg for cerebellar particulate protein; β_I and β_{II} were linear in the range 10–30 μg. Mutant and wild-type comparisons were performed at 50 μg of protein for the γ isotype, 20 μg for the α isotype, and 10 μg for the β isotypes. Molecular weight standards and an extract from whole brain, which served as a quantitative standard for comparisons, were also added to each gel. After electrophoresis, protein was transferred by electroblotting to nitrocellulose filters overnight at 4°C. The filters were stained with Ponceau S to verify transfer and then the blot was incubated in TBS-T (20 mM Tris Cl, pH 7.6/137 mM NaCl/0.1% Tween 20) containing 0.1% NaN_3 and 5% dry milk for 1 hr. Primary antibody (1:500) was added in the identical buffer for 3 hr and incubated with shaking at room temperature. Anti-PKCγ antibody was purchased from GIBCO/BRL, and anti-PKCα_1, -β_I, and -β_{II} antibodies were obtained from Santa Cruz Biotechnology. The filters were washed in the same buffer without antibody and then incubated with ^{125}I-labeled protein A (200,000 dpm/ml) (DuPont/NEN) for 2 hr and extensively washed in TBS-T. Filters were dried and placed on Hyperfilm-γ (Amersham) at −70°C for 3–7 days. To quantify each sample, the filter was cut by using the autoradiograph as a guide, radioactivity was measured in a γ counter (Beckman LS 1800), the filter background counts were subtracted, and each sample was expressed as the percent of whole brain standard.

$^{36}Cl^-$ Uptake Assay. Isolated brain membrane vesicles (microsacs) were prepared, and muscimol-stimulated $^{36}Cl^-$ uptake was assayed (16). The tissue was homogenized in 4.5 ml of ice-cold assay buffer (145 mM NaCl/5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/10 mM D-glucose/10 mM Hepes, adjusted to pH 7.5 with Tris base). The final pellet was suspended in assay buffer, and tissue aliquots (0.6–0.8 mg of protein) were incubated in a 34°C water bath for 5 min. Uptake was initiated by adding 200 μl of $^{36}Cl^-$ solution (2 μCi/ml of assay buffer; 1 μCi = 37 kBq) containing the drugs to be tested. Three seconds after the addition of $^{36}Cl^-$, influx was terminated by the addition of 4 ml of ice-cold quench buffer (assay buffer with 0.1 mM picrotoxin and 0.1 mM bicuculline methiodide) and rapid filtration and washing. The amount of $^{36}Cl^-$ bound to the filters in the absence of membranes (no-tissue blank) was subtracted from all values. Muscimol-dependent influx was defined as the amount of $^{36}Cl^-$ taken up while agonist was present in the medium (total uptake) minus the amount of $^{36}Cl^-$ taken up when agonist was not present (muscimol-independent or nonspecific uptake). The investigator performing the $^{36}Cl^-$ uptake studies (S.J.M.) was not aware of the genotype of the mice.

RESULTS

To assure that the mutation abolished PKCγ and to determine whether it affected the levels of other isoforms of PKC, we carried out quantitative immunoblot analysis using cerebellar tissue (Fig. 1). The mutation eliminated PKCγ without any detectable changes in the other three isoforms, confirming the utility of these mice for studying the role of PKCγ in cerebellar function.

Cerebellar and cortical membranes were prepared from wild-type and null mutant mice for the study of GABA-

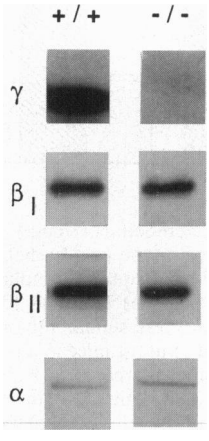

FIG. 1. Immunoblot determination of PKC protein levels in cerebellum from wild-type (+/+) and null mutant (−/−) mice.

activated Cl^- channel function. Stimulation of $^{36}Cl^-$ uptake by the $GABA_A$ agonist muscimol was normal for the heterozygous and homozygous null mutant animals (Fig. 2). The PKCγ mutation did not alter the basal uptake of $^{36}Cl^-$; similar results for basal and muscimol-stimulated uptake were obtained with membrane vesicles from cerebellum and cortex (Fig. 2) as well as midbrain (data not shown). In contrast to these results, enhancement of muscimol action by ethanol (15 mM) was abolished in the null mutant animals (Fig. 3). These results from cerebellum are particularly illuminating because only the dendrites of Purkinje cells of cerebellum contain PKCγ (21–23), yet the mutation completely eliminated the action of ethanol. Thus, all ethanol-sensitive $GABA_A$ receptors of cerebellum must be located on Purkinje dendrites. As in cerebellum and cortex, ethanol (15 mM) enhanced muscimol actions with membranes from the midbrain region of wild-type mice but produced no enhancement in null mutant mice (data not shown).

Like ethanol, flunitrazepam and pentobarbital enhance GABAergic function, but, unlike ethanol, these drugs were equally effective at enhancing $GABA_A$ receptor function in membranes prepared from cerebellum or cortex (Fig. 4) of wild-type and null mutant animals. When drug actions on cerebellar $GABA_A$ receptors were compared, flunitrazepam (1 μM) or pentobarbital (30 μM) produced 25–40% larger enhancement of muscimol action than was obtained with 15 μM ethanol. Thus, ethanol produced almost as much augmentation of $GABA_A$ receptor function as a benzodiazepine and a barbiturate known to produce their behavioral actions by enhancing GABA action. This is consistent with other $^{36}Cl^-$ flux studies (1–3).

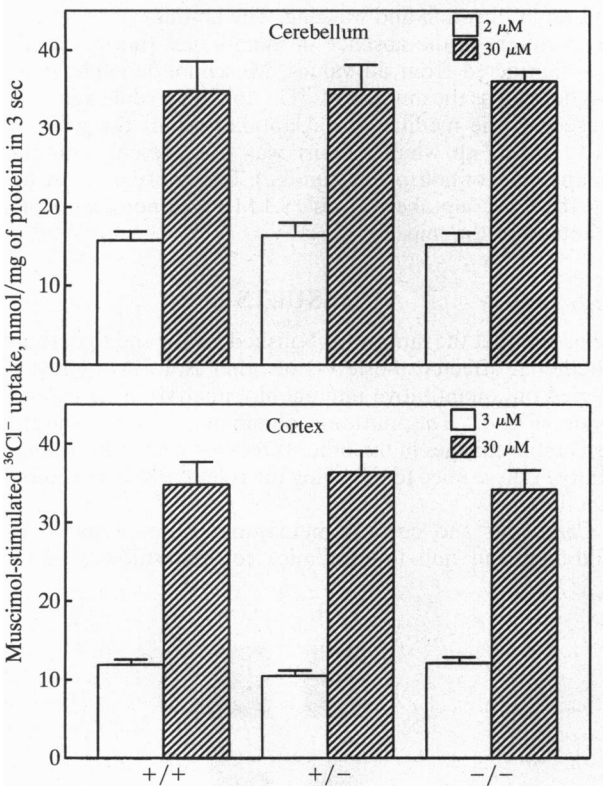

FIG. 2. Function of cerebellar (*Upper*) and cortical (*Lower*) $GABA_A$ receptors from wild-type (+/+), heterozygous (+/−), and null mutant (−/−) mice. $^{36}Cl^-$ uptake stimulated by either submaximal (2 or 3 μM, open bars) or maximal (30 μM, hatched bars) concentrations of muscimol was similar for preparations from wild-type, heterozygous, or null mutant mice. Values are mean ± SEM; n = 32–34 for 2 μM muscimol in cerebellum, n = 18 for 3 μM muscimol in cortex, and n = 4–6 for 30 μM muscimol in both brain regions. Basal uptake of ^{36}Cl (nmol/mg of protein in 3 sec) was 20 ± 1 for wild type, 21 ± 1 for heterozygous mice, and 20 ± 1 for null mutant mice in cerebellum and 19 ± 1 for wild type, 20 ± 1 for heterozygous mice, and 19 ± 1 for null mutant mice in cortex.

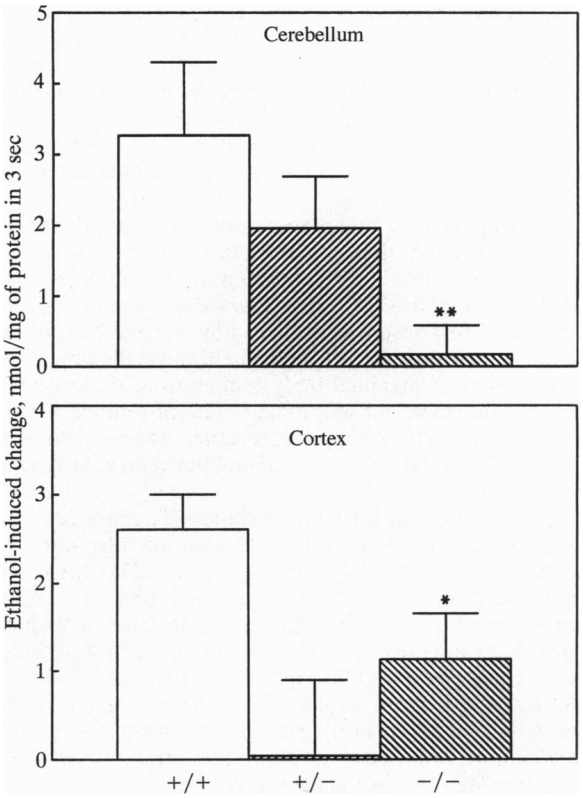

FIG. 3. Enhancement of $GABA_A$ receptor function by ethanol is reduced by deletion of PKCγ, as shown by $^{36}Cl^-$ uptake in cerebellar (*Upper*) and cortical (*Lower*) membrane preparations from wild-type (+/+), heterozygous (+/−), and null mutant (−/−) mice. Ethanol-induced changes were calculated by subtracting the control muscimol-dependent $^{36}Cl^-$ uptake from the muscimol-dependent $^{36}Cl^-$ uptake measured in the presence of ethanol. Muscimol concentration was 2 μM for cerebellum and 3 μM for cortex; ethanol was 15 mM. Values are mean ± SEM, n = 10–12 for cerebellum and 7–19 for cortex. Effects of ethanol were dependent upon genotype [$F(2, 32) = 4.4$, $P < 0.02$ for cerebellum; $F(2, 41) = 4.6$, $P < 0.02$ for cortex] and differed between wild-type and homozygous mutant groups (**, $P < 0.01$, cerebellum; *, $P < 0.02$, cortex).

Behavioral studies were consistent with these neurochemical results. The loss of righting reflex and the hypothermia produced by administration of ethanol were reduced in the null mutants as compared with wild-type mice (Figs. 5 and 6). However, the hypothermia produced by flunitrazepam or pentobarbital was not different in the null mutants (Fig. 6). In addition, the duration of loss of righting reflex produced by pentobarbital was the same for wild-type and null mutant mice (Fig. 5). The reduced effects of ethanol *in vivo* were not due to changes in metabolism or distribution of the drug in the mutant animals. Following injection of ethanol (3.5 g/kg, i.p.), the peak blood ethanol concentrations were 422 ± 22 and 398 ± 45 mg/dl in wild-type and mutant mice, respectively. The ethanol clearance rates were 1.88 ± 0.25 and 1.36 ± 0.15 mg/dl per minute in wild-type and mutant mice, respectively.

DISCUSSION

These results provide evidence that elimination of a specific kinase affects the function of a ligand-gated ion channel and alters drug sensitivity *in vivo*. Further, our findings emphasize the unique interactions of ethanol with the $GABA_A$ receptor

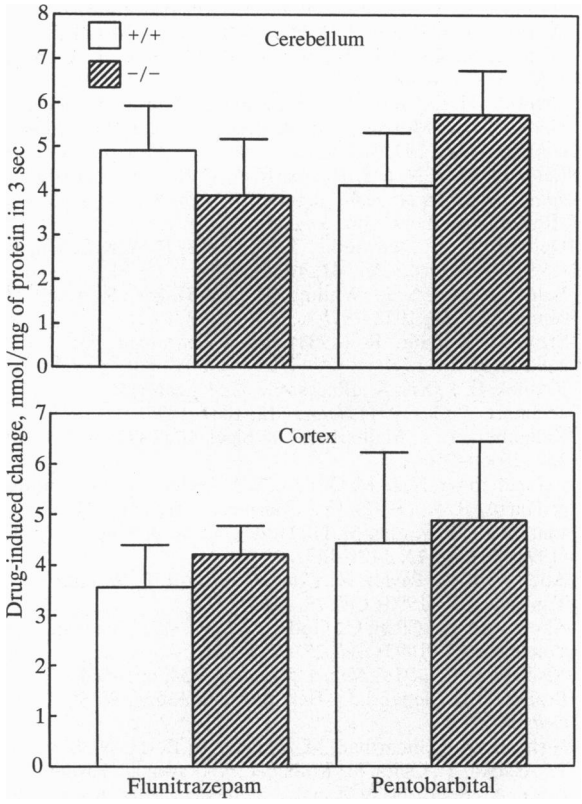

FIG. 4. Enhancement of $GABA_A$ receptor function by flunitrazepam or pentobarbital is not affected by PKCγ deletion, as shown by $^{36}Cl^-$ uptake in cerebellar (*Upper*) or cortical (*Lower*) membranes from wild-type (+/+, open bars) and null mutant (−/−, hatched bars) mice. Drug-induced changes were calculated by subtracting the control muscimol-dependent $^{36}Cl^-$ uptake from the muscimol-dependent $^{36}Cl^-$ uptake measured in the presence of drug. Muscimol concentrations were 2 μM for cerebellum and 3 μM for cortex; flunitrazepam was 1 μM for cerebellum and 0.1 μM for cortex, and pentobarbital was 30 μM for cerebellum and 10 μM for cortex. Values are mean ± SEM, n = 10–12.

by showing that PKC is a critical determinant of ethanol actions *in vivo* and *in vitro* without altering the actions of other activators/modulators of GABA-activated Cl^- channels such as muscimol, flunitrazepam, and pentobarbital. This is consistent with data from recombinant receptors showing that deletion of a phosphorylation site in the γ2L subunit of the $GABA_A$ receptor reduces the action of ethanol without altering the effects of benzodiazepines or pentobarbital (7) and with animal studies showing that selection of mice for differences in ethanol sensitivity does not necessarily produce differences in behavioral sensitivity to benzodiazepines or barbiturates (30, 31). However, the lack of effect of the deletion of PKCγ on muscimol action was unexpected in view of studies of recombinant receptors showing both inhibition and enhancement of GABA action by activation of PKC (13–17), although normal GABAergic function is consistent with the unremarkable behavior of the mutant mice. It is possible that remaining PKC isozymes provide sufficient kinase activity to regulate $GABA_A$ receptors.

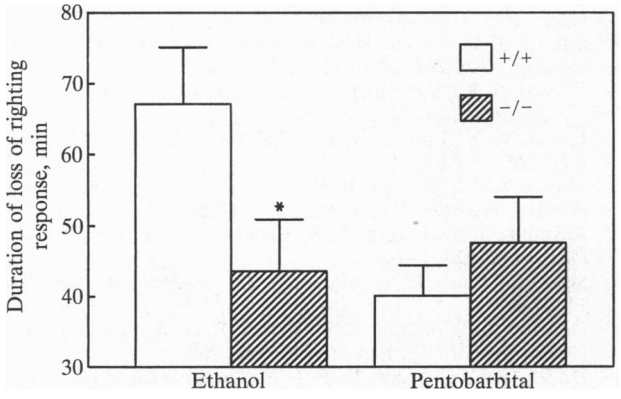

FIG. 5. Behavioral effects of ethanol, flunitrazepam, or pentobarbital in wild-type (+/+, open bars) and PKCγ null mutant (−/−, hatched bars) mice. Sensitivity to ethanol and pentobarbital was measured by the duration of the loss of righting reflex; ethanol (3.5 g/kg) or pentobarbital (62 mg/kg) was administered by i.p. injection. Each value is the mean ± SEM. Mutant mice were less sensitive to ethanol [$t(17) = 2.3$; *, $P < 0.05$; n = 7 or 8] but wild-type and mutant mice were not differently sensitive to pentobarbital (n = 10).

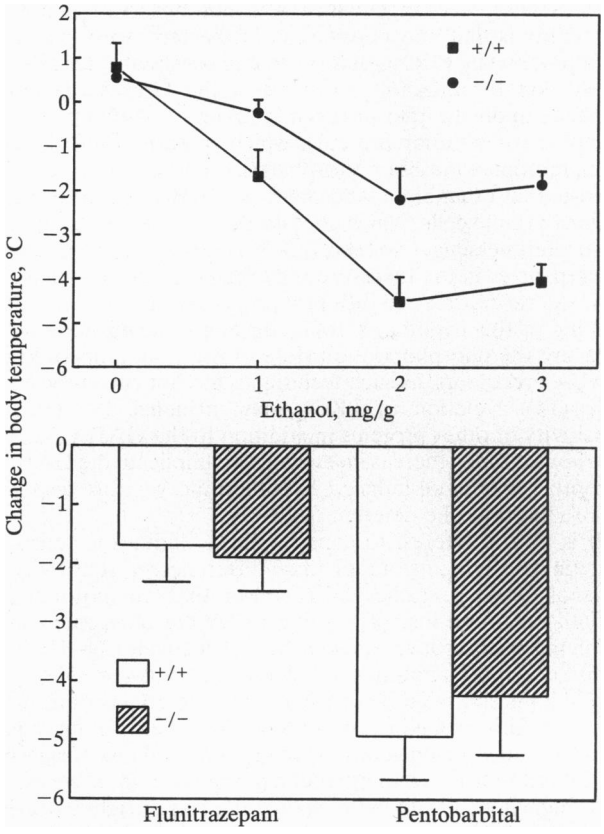

FIG. 6. Sensitivity of mutant mice to hypothermia produced by ethanol, pentobarbital, or flunitrazepam. (*Upper*) Ethanol-induced hypothermia. Alterations of body temperature were measured in wild-type (+/+, ■) and PKCγ null mutant (−/−, ●) mice at 30 min after an i.p. injection of ethanol. Data are presented as the change in body temperature relative to basal temperatures recorded 5 min prior to drug administration. PKCγ null mutants are less sensitive than wild-type mice [$F(1, 9) = 11$, $P < 0.01$], but both genotypes show decreased body temperature as a function of dose [$F(3, 27) = 53$, $P <$ 0.0001]. Each point represents the mean ± SEM (n = 5 or 6 mice). (*Lower*) Flunitrazepam- and pentobarbital-induced hypothermia. Alterations in body temperature were measured at the time of peak hypothermia. This was 60 min after administration of flunitrazepam (100 mg/kg) or 30 min after administration of pentobarbital (50 mg/kg). Wild-type (+/+, open bars) and PKCγ null mutant (−/−, hatched bars) mice did not differ in time of maximal depression (data not shown) or magnitude of response. Each point is the mean ± SEM (n = 10 or 11 mice).

There are several possible mechanisms for actions of ethanol on the $GABA_A$ receptor. One is that ethanol alters the activity of PKCγ, which in turn alters the action of GABA. However, this would require modulation of the receptor by PKCγ; if this were the case, deletion of PKCγ should have altered the action

of muscimol, a change which was not observed. Another possibility is that phosphorylation of the receptor (or associated protein) by PKCγ confers ethanol sensitivity. This would imply that the ethanol sensitivity of the $GABA_A$ receptor depends upon the phosphorylation state of a subunit of the receptor (or another protein), which in turn could depend upon multiple kinases or phosphatases. This is consistent with reports that ethanol enhancement of GABA action on cerebellar Purkinje cells (which contain PKCγ) requires activation of second-messenger systems (32–36) and provides a basis for discrepancies in the literature regarding ethanol sensitivity of $GABA_A$ receptors from different preparations (10, 11, 37, 38). Testing of this hypothesis awaits development of methods to measure the phosphorylation state of the γ subunits of brain $GABA_A$ receptors, as such techniques are not currently available (14). Deletion of PKCγ may influence the ethanol sensitivity of other proteins in addition to the $GABA_A$ receptor. For example, there is no evidence to implicate the $GABA_A$ receptor in ethanol-induced hypothermia, yet this response was reduced by the deletion of PKCγ.

It is also of interest to consider these findings in terms of genetic determinants of individual differences in sensitivity to ethanol. Animal studies have shown that ethanol-induced hypothermia and loss of righting reflex are polygenic traits, involving at least four and seven loci, respectively (39–41). The difference in ethanol-induced sleep time between wild-type and null mutant mice is consistent with the effects of a single gene on this behavior. Assuming that there is an equal contribution of seven genes to ethanol-induced loss of righting reflex and four genes to ethanol hypothermia, then the effect of a single gene would be to change the loss of righting reflex by 10 min (3.5-mg/g dose) and hypothermia by 1.1°C (2-mg/g dose), as shown with data from Figs. 5 and 6. The effect of PKCγ deletion was to change loss of righting reflex by 23 min and hypothermia by 2.3°C. This is consistent with the action of one major gene that exerts twice the average effect on the phenotypes. This suggests PKCγ as a candidate gene (or all PKCs as a gene family) that may determine genetic differences in ethanol sensitivity. This is of potential importance in the search for genetic determinants of alcoholism, because individuals at genetic risk for development of alcoholism (i.e., those with a family history of the disease) appear to differ in acute sensitivity to ethanol (42–44).

In summary, these results provide the most compelling evidence to date that PKC isoenzymes are important mediators of the behavioral effects of ethanol and the effects of this drug on the $GABA_A$ receptor. It will be of interest to evaluate these candidate genes in populations differing in ethanol sensitivity and alcoholism.

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