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$GABA_A$ Receptor Function in the Cerebral Cortex of Alcohol-Naive P and NP Rats

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THIELEN, R. J., W. J. MCBRIDE, L. LUMENG AND T.-K. LI. *$GABA_A$ receptor function in the cerebral cortex of alcohol-naive P and NP rats*. PHARMACOL BIOCHEM BEHAV **59**(1) 209–214, 1998.—Previous studies have demonstrated an innate difference in the sensitivity of ethanol-naive P and NP rats to the acute intoxicating effects of high doses of ethanol. A number of studies have suggested that the acute intoxicating effects of ethanol may be mediated in part through potentiation of $GABA_A$/benzodiazepine receptor function. In the present study, the function of $GABA_A$/benzodiazepine receptors was studied in ethanol-naive alcohol-preferring (P) and -nonpreferring (NP) lines of rats by measuring $^{36}Cl^-$ influx into cortical microsacs. GABA, in a concentration-dependent manner, increased $^{36}Cl^-$ influx to an equivalent extent into cortical microsacs from P and NP rats (EC_{50} = 9.0 ± 1.0 and 10 ± 1.1 μM; E_{max} = 30.8 ± 1.3 and 28.1 ± 0.9 nmol Cl^-/mg protein/3 s, respectively). Pentobarbital (30 μM) enhanced GABA-stimulated $^{36}Cl^-$ uptake (75 and 71% increase for P and NP rats, respectively) equally well in cortical microsacs from P and NP rats. Likewise, phenobarbital potentiation of GABA-stimulated $^{36}Cl^-$ influx was similar in cortical microsacs from P and NP rats. Phenobarbital, at the highest concentration tested (3 mM), directly stimulated $^{36}Cl^-$ influx to a similar extent in P and NP rats. However, ethanol failed to alter GABA-stimulated $^{36}Cl^-$ uptake into cortical microsacs prepared from ethanol-naive P and NP rats. The present results suggest that the differences between P and NP rats in innate sensitivity to the high dose effects of ethanol do not appear to be due to differences in cortical $GABA_A$ receptor function. © 1998 Elsevier Science Inc.

$GABA_A$ receptors Chloride influx Alcohol-preferring rats Pentobarbital Phenobarbital

ETHANOL, at physiologically relevant concentrations, can potentiate $GABA_A$/ benzodiazepine receptor function measured in vitro. Ethanol can enhance GABA-stimulated Cl^- currents measured in cultured neurons from the rat dorsal root ganglion (30) and mouse cortex and hippocampus (1,37). Additionally, ethanol has been shown to potentiate GABA- and muscimol-stimulated $^{36}Cl^-$ uptake into membrane preparations from the cerebral cortex and cerebellum of mice (10) and rats (32).

The long-sleep (LS) and short-sleep (SS) mice were selectively bred for differential sensitivity to the hypnotic effects of ethanol as measured by the duration of loss of righting reflex or "sleep-time" (24). In addition to being more sensitive to the acute intoxicating effects of ethanol, LS mice also show longer duration of loss of righting reflex to a number of CNS depressants, including benzodiazepines and some barbiturates, i.e., phenobarbital (22,23,25), but not other barbiturates, i.e., pentobarbital (31). Differences in $GABA_A$/benzodiazepine receptor function between LS and SS mice, as measured by $^{36}Cl^-$ influx into cortical microsacs in response to stimulation of $GABA_A$/benzodiazepine receptor function by agonists (3), as well as potentiation of agonist effects by ethanol (3), benzodiazepines (10), and phenobarbital (4), suggest that differences in the $GABA_A$/benzodiazepine receptor might be partially responsible for the observed behavioral differences.

The alcohol-preferring (P) and -nonpreferring (NP) rats were selectively bred for differences in alcohol-seeking behavior (15). In addition to ethanol preference, P and NP rats also differ in response to systemic administration of ethanol (21,36). If differences in the sensitivity of P and NP rats to the motor impairing effects of an acute, intoxicating effect of ethanol are a result, in part, to innate differences in the $GABA_A$ receptor between P and NP rats, then this might be detected by measuring $GABA_A$ receptor function in vitro. The present studies were carried out to determine if differences exist in

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$GABA_A$ receptor function between P and NP rats, as measured by GABA-stimulated $^{36}Cl^-$ influx, and barbiturate and ethanol potentiation of GABA-stimulated $^{36}Cl^-$ influx, into cortical microsacs.

METHOD

Animals

Alcohol-naive, male P and NP rats (70–120 days) from the S29–S35 generations (17,20) were mainly used in this study. A small number of experiments were conducted with alcohol-naive, male high alcohol-drinking (HAD) and low alcohol-drinking (LAD) lines of rats (70–120 days old), from the S12–S17 generations (17), to test the effects of ethanol on $^{36}Cl^-$ influx. Animals were housed in a temperature controlled room, maintained on a 12 L:12 D cycle (lights on at 0400 h), with food and water available ad lib. Animals were housed individually in standard plastic animal containers. All rats were handled daily and habituated to the guillotine for at least 1 week prior to each experiment. The animals were killed by decapitation. Microsacs were prepared and $^{36}Cl^-$ influx was measured for both lines in the same assay.

Chemicals

NaCl, $MgCl_2$ (anhydrous), 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), Tris[hydroxymethyl]aminomethane (Tris), GABA, pentobarbital (sodium salt), phenobarbital (sodium salt), and picrotoxin were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY). D-Glucose was purchased from MCB Reagents (Gibbstown, NJ). KCl was purchased from Mallinckrodt (St. Louis, MO). $CaCl_2{\cdot}2H_2O$ was purchased from J. T. Baker Chemical Co. (Pillipsburg, NJ). $^{36}Cl^-$($Na^{36}Cl$, 8–14 mCi/g Cl) was acquired from ICN (Irvine, CA). Deionized, glass-distilled H_2O was used to make all aqueous solutions.

Microsac Preparation

Microsacs were prepared by the method of Harris and Allen (9). The cerebral cortex was carefully dissected on ice and homogenized (10–12 strokes) using a glass-Teflon homogenizer (Thomas, size C) in 4.5 ml of ice-cold buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 10 mM HEPES adjusted to pH 7.5 with Tris base). The homogenate was centrifuged at 900 × g for 15 min at 4°C. The supernatant was decanted and the pellet resuspended in 8 ml of ice-cold buffer. The resulting suspension was then centrifuged at 900 × g for 15 min at 4°C. The final pellet was resuspended in 7 ml of ice-cold buffer to give a protein concentration of approximately 5–7 mg protein/ml. Protein concentration was determined by the method of Lowry et al. (19).

$^{36}Cl^-$ Influx Assay

Influx of $^{36}Cl^-$ into microsacs was performed as described by Harris and Allan (9). The microsac suspension (200 μl) was preincubated for 2 min at 34°C in a shaking water bath (11). $^{36}Cl^-$ (1.6 μCi/ml) in buffer (200 μl) containing various concentrations of the test agents, preincubated to 34°C, was added to the microsac suspensions to initiate uptake. Uptake was terminated 3 s later by the addition of 4 ml ice-cold buffer containing 100 μM picrotoxin followed by rapid filtration under vacuum (254 mmHg) through glass microfibre filters (Whatman GF/C, Whatman Lab Sales, Hillsboro, OR) using a Hoeffer filter manifold (model FH 224V, San Francisco, CA). After removing the towers, the filters were washed with 8 ml of ice-cold buffer containing 100 μM picrotoxin. The filters were transferred to scintillation vials containing 10 ml of scintillation cocktail (CytoScint, ICN, Irvine, CA) and the amount of $^{36}Cl^-$ taken up was determined by liquid scintillation spectrophotometric methods. The amount of $^{36}C^-$ bound to the filters in the absence of cortical membranes was subtracted from all values.

Statistical Analysis

All data are expressed as the means ± standard error of the mean (SEM). The maximal effect (E_{max}) and half-maximal effective concentration (EC_{50}) values were determined by fitting the data to a three-parameter logistic function using the nonlinear regression algorithm of Sigmaplot, ver. 5.0 (Jandel Scientific, San Rafael, CA). Fitting the data to a four-parameter logistic function did not provide a significant improvement in the fit of the data to the curve. The data were analyzed by analysis of variance (ANOVA) followed by post hoc Newman–Keuls test, when multiple comparisons were carried out, using CCS: Statistica (ver. 3.1) (StatSoft, Inc., Tulsa, OK). Student's two-tailed independent *t*-test was used when individual comparisons between pairs of means were carried out (CSS: Statistica, ver. 3.1).

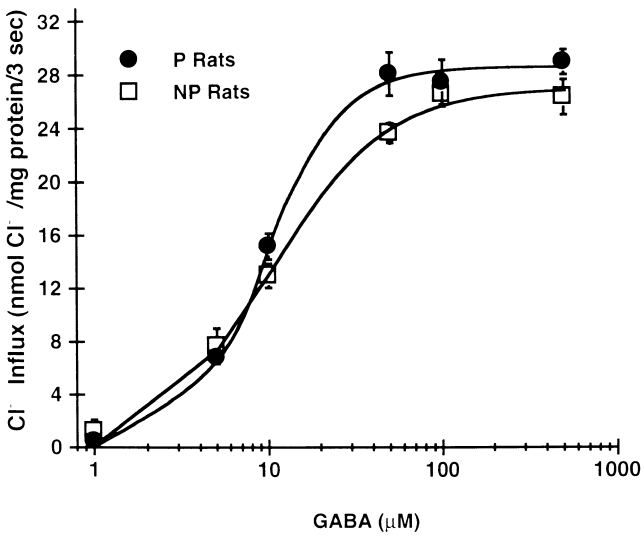

FIG. 1. Effect of GABA on $^{36}Cl^-$ influx into cortical microsacs from P and NP rats. The data represent GABA-dependent $^{36}Cl^-$ influx (mean ± SEM) (n = 4, P; n = 5, NP). GABA-dependent $^{36}Cl^-$ influx was defined as the amount of $^{36}Cl^-$ influx in the presence of GABA minus $^{36}Cl^-$ influx in the absence of GABA (GABA-independent influx). GABA-independent $^{36}Cl^-$ influx was 27.2 ± 1.3 and 24.6 ± 0.9 nmol Cl^-/mg protein/3 s for P and NP rats, respectively; there was no significant difference between the lines. There was a significant effect of GABA concentration, $F(5, 35) = 249$, $p < 0.001$, but not of line or line × GABA concentration interaction. The EC_{50} values for GABA-stimulated $^{36}Cl^-$ influx were 9.0 ± 1.0 and 10.0 ± 1.1 μM for P and NP rats, respectively; there was no significant difference between the lines. The E_{max} values were 30.8 ± 1.3 and 28.1 ± 0.9 nmol Cl^-/mg protein/3 s for P and NP rats, respectively; there was no significant difference between the lines.

RESULTS

There was no significant difference in GABA-independent $^{36}Cl^-$ influx between P and NP rats (27.2 ± 1.3 and 24.6 ± 0.9 nmol Cl^-/mg protein/3 s, respectively; n = 4–5). GABA (1–500 μM) stimulated $^{36}Cl^-$ influx, in a concentration-dependent manner, into cortical microsacs prepared from P and NP rats, $F(5, 35) = 249$, $p < 0.001$ (Fig. 1). The effect of GABA (1–500 μM) on $^{36}Cl^-$ influx into cortical microsacs was the same in P and NP rats (Fig. 1). There was no difference in the EC_{50} values for GABA-stimulated $^{36}Cl^-$ influx into cortical microsacs from alcohol-naive P and NP rats (9.0 ± 1.0 and 10.0 ± 1.1 μM, respectively). The E_{max} values were also similar for P and NP rats (30.8 ± 1.3 and 28.1 ± 0.9 nmol Cl^-/mg protein/3 s, respectively).

To determine if the potentiation of GABA-stimulated $^{36}Cl^-$ influx by barbiturates was similar between P and NP rats, the effect of pentobarbital was determined (Fig. 2). Pentobarbital (30 μM) did not significantly alter GABA-independent $^{36}Cl^-$ influx in either P (27 ± 1 nmol Cl^-/mg prot/3 s) or NP (27 ± 2 nmol Cl^-/mg prot/3 s) rats. Pentobarbital (30 μM) did, however, enhance 5 μM GABA-stimulated $^{36}Cl^-$ influx in cortical microsacs prepared from P and NP rats, $F(1, 10) = 59.4$, $p < 0.001$, main effect of 5 μM GABA + 30 μM pentobarbital, $p < 0.005$ vs. 5 μM GABA alone by post hoc Newman–Keuls. However, there was no significant difference in the effects of pentobarbital between P and NP rats (Fig. 2).

Because it was previously demonstrated that differences in phenobarbital-enhancement of agonist stimulated $^{36}Cl^-$ influx between LS and SS mice appears to be associated with differences in ethanol sensitivity (4), the effect of phenobarbital on GABA-stimulated $^{36}Cl^-$ influx into cortical microsacs prepared from P and NP rats was also determined (Fig. 3). Phenobarbital (0.1–3.0 mM) enhanced GABA-stimulated $^{36}Cl^-$ influx into cortical microsacs prepared from P and NP rats in a concentration-dependent manner, $F(5, 40) = 99.7$, $p < 0.001$. Comparison of phenobarbital-enhancement of 5 μM GABA-stimulated $^{36}Cl^-$ influx did not reveal any significant differences between P and NP rats. Phenobarbital did not appear to have maximally enhanced GABA-stimulated $^{36}Cl^-$ influx at the highest concentration tested, 3 mM (Fig. 3); therefore, E_{max} and EC_{50} values were not determined. When tested alone, phenobarbital directly stimulated $^{36}Cl^-$ influx into cortical microsacs from P and NP rats, $F(3, 18) = 9.21$, $p < 0.001$, but only at the highest concentration tested (3 mM), $p < 0.05$ (Fig. 4). There was no difference between P and NP rats in the ability of phenobarbital by itself to stimulate $^{36}Cl^-$ influx into cortical microsacs.

Ethanol (1–90 mM) did not enhance 5 μM GABA-stimulated $^{36}Cl^-$ influx in cortical microsacs from P and NP rats (Fig. 5). To evaluate possible strain differences in the effects of ethanol on Cl^- influx, experiments were also carried out with the HAD and LAD lines of rats. Ethanol (1–90 mM) failed to alter 5 μM GABA-stimulated $^{36}Cl^-$ influx into cortical microsacs prepared from HAD (n = 5) and LAD (n = 6) rats (data not shown but are similar to results given in Fig. 5 for P and NP rats). There was no significant line difference between the HAD and LAD rats in either basal $^{36}Cl^-$ influx (26 ± 2 for HAD vs. 26 ± 1 nmol Cl^-/mg prot/3 s for LAD rats) or 5 μM GABA-stimulated Cl^- influx (33 ± 2 for HAD vs. 36 ± 1 nmol Cl^-/mg prot/3 s for LAD rats).

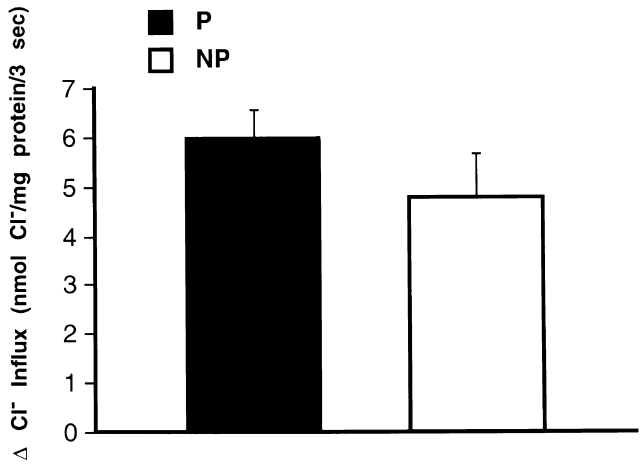

FIG. 2. Effect of 30 μM pentobarbital on GABA-dependent $^{36}Cl^-$ influx into cortical microsacs prepared from P and NP rats. GABA-independent $^{36}Cl^-$ influx was subtracted from all values. GABA-independent $^{36}Cl^-$ influx was 27 ± 1 and 27 ± 2 nmol Cl^-/mg prot/3 s in the P and NP rats, respectively. The data represent pentobarbital-enhanced $^{36}Cl^-$ influx (mean ± SEM; n = 6, P; n = 6, NP). Pentobarbital-enhanced $^{36}Cl^-$ influx is defined as the amount of $^{36}Cl^-$ influx in the presence of pentobarbital and 5 μM GABA minus 5 μM GABA-dependent $^{36}Cl^-$ influx when pentobarbital was absent. GABA-dependent influx was 7.6 ± 0.5 and 6.8 ± 1.2 nmol Cl^-/mg prot/3 s in P and NP rats, respectively; these values were not statistically different. There was a significant effect of pentobarbital on GABA-stimulated Cl^- influx, $F(1, 10) = 59.4$, $p < 0.001$, but no significant line effect or line × treatment interaction.

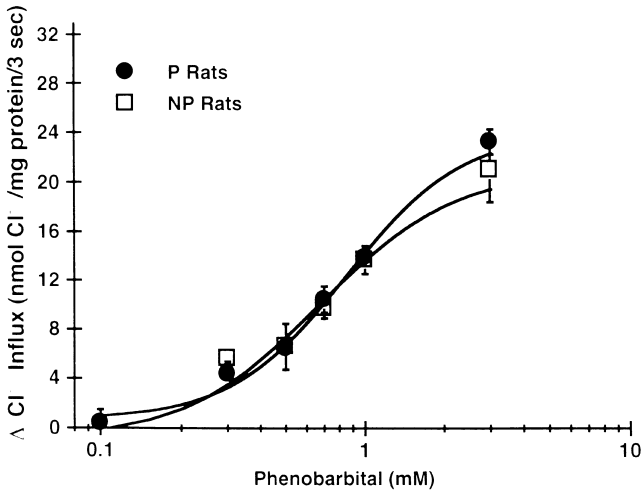

FIG. 3. Effect of phenobarbital on 5 μM GABA-stimulated $^{36}Cl^-$influx into cortical microsacs prepared from P and NP rats. GABA-independent $^{36}Cl^-$ influx was subtracted from all values. GABA-independent $^{36}Cl^-$ influx was 25.8 ± 0.8 and 24.7 ± 0.9 nmol Cl^-/mg protein/3 s in the P and NP rats, respectively; there was no significant difference between the lines. Data represent phenobarbital-enhanced $^{36}Cl^-$ influx (mean ± SEM; n = 6). Phenobarbital-enhanced $^{36}Cl^-$ influx was defined as the amount of $^{36}Cl^-$ influx in the presence of phenobarbital and 5 μM GABA minus 5 μM GABA-dependent $^{36}Cl^-$ influx when phenobarbital was absent. 5 μM GABA-dependent $^{36}Cl^-$ influx was 6.8 ± 1.1 and 8.6 ± 0.2 nmol Cl^-/mg protein/3 s in P and NP rats, respectively; there was no significant difference between the lines. There was a significant effect of phenobarbital concentration, $F(5, 40) = 99.7$, $p < 0.001$, but no line effect or line × phenobarbital concentration interaction.

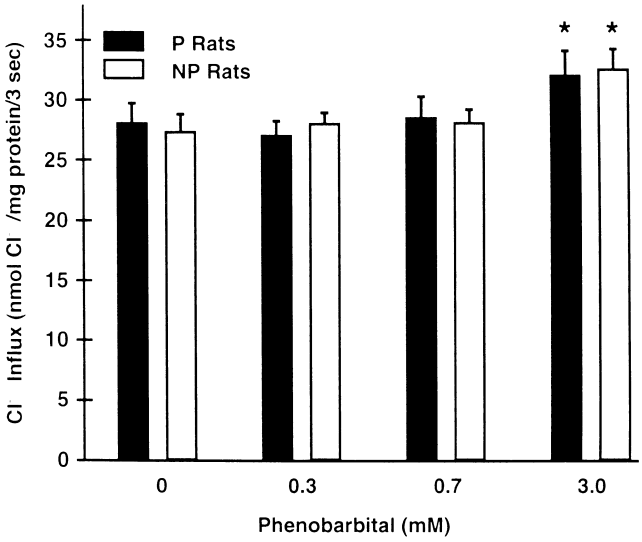

FIG. 4. Effect of phenobarbital on $^{36}Cl^-$ influx into cortical microsacs from P and NP rats. The data represent $^{36}Cl^-$ influx (mean ± SEM; $n = 4$). There was a significant effect of phenobarbital concentration, $F(3, 18) = 9.21$, $p < 0.001$; but, no effect of line or line × phenobarbital concentration interaction. $*p < 0.05$ compared to phenobarbital-independent $^{36}Cl^-$influx in the same line, by post hoc Newman–Keuls.

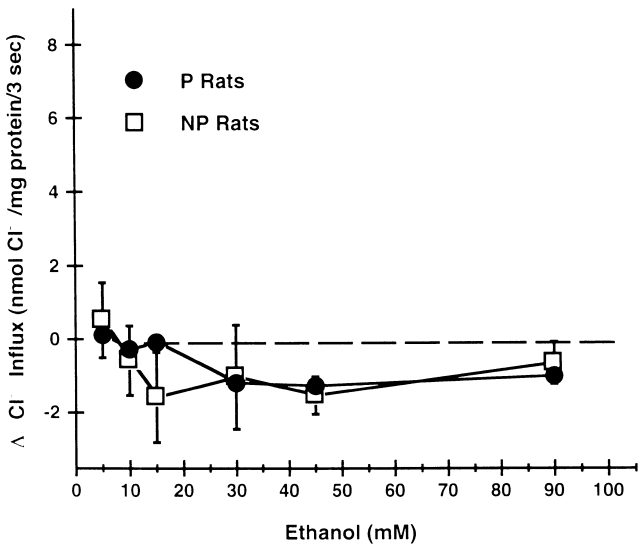

FIG. 5. The effect of ethanol on 5 μM GABA-stimulated $^{36}Cl^-$influx into cortical microsacs from P and NP rats. Data represent ethanol-enhanced $^{36}Cl^-$ influx (mean ± SEM; $n = 5$). GABA-independent $^{36}Cl^-$ influx was subtracted from all values. GABA-independent $^{36}Cl^-$ influx was 25.4 ± 1.0 and 25.3 ± 1.5 nmol Cl^-/mg protein/3 s in the P and NP rats, respectively. There was no significant difference between the lines in GABA-independent $^{36}Cl^-$influx. Ethanol-enhanced $^{36}Cl^-$ influx was defined as the amount of $^{36}Cl^-$ influx in the presence of ethanol and 5 μM GABA minus 5 μM GABA-dependent $^{36}Cl^-$ influx when ethanol was absent. 5 μM GABA-dependent $^{36}Cl^-$ influx was 9.2 ± 0.6 and 10.8 ± 0.8 nmol Cl^-/mg protein/3 s for P and NP rats, respectively. There was no significant difference between the lines in GABA-dependent $^{36}Cl^-$ influx. There was no significant effect of line, ethanol concentration, or line × ethanol concentration interaction.

DISCUSSION

It was disappointing to find in this study that ethanol did not potentiate GABA-stimulated Cl^- influx into cortical microsacs from the P and NP (Fig. 5) or HAD and LAD lines. Because NP rats, compared to P rats, are more sensitive to the acute intoxicating effects of ethanol, it was anticipated that ethanol would potentiate GABA-stimulated Cl^- influx into cortical microsacs from the NP line. In mice and rats selectively bred to be sensitive to the acute intoxicating effect of ethanol (LS mice and HAS rats), ethanol was shown to enhance GABA agonist-stimulated Cl^- uptake into cortical microsacs (5,10).

The failure to observe ethanol potentiation of GABA-stimulated $^{36}Cl^-$ influx was not a result of the strain of rat used. Ethanol not only failed to enhance $^{36}Cl^-$ influx when examined in microsacs from P and NP rats, which were derived from Wistar stock (15), but also failed to enhance GABA-stimulated $^{36}Cl^-$ uptake into cortical microsacs from HAD and LAD rats, which were derived from the N/Nih heterogeneous rats (16), the same stock that was used to derive the HAS and LAS rats (8).

Other investigators have reported that ethanol, alone or in combination with $GABA_A$ receptor agonists, failed to alter $^{36}Cl^-$ influx (27,28). In both of these studies, $GABA_A$ receptor agonists stimulated $^{36}Cl^-$ influx in a concentration-dependent manner (27,28); this GABA agonist effect could be blocked by the noncompetitive $GABA_A$ receptor antagonist picrotoxin (28). In addition, these investigators demonstrated pentobarbital enhancement of agonist-stimulated $^{36}Cl^-$ influx (27,28), as well as diazepam enhancement of GABA-mediated $^{36}Cl^-$ influx (27). In the present study, GABA also stimulated Cl^- influx into cortical microsacs from both P and NP rats (Fig. 1). This GABA-stimulated influx was enhanced by pentobarbital (Fig. 2) and phenobarbital (Fig. 3), as well as by flunitrazepam (34).

Electrophysiological experiments also have not demonstrated consistent ethanol effects at the $GABA_A$, receptor. Some studies showed that ethanol potentiated GABA-mediated responses, whereas others showed no effect of ethanol on GABA-mediated responses in cultured cells from the same tissue (30,37). Indeed, a number of reviews examining the evidence concerning ethanol effects on GABA-mediated responses have emphasized the inconsistency in the data [see (14,18)]. The results of biochemical studies, examining ethanol effects on $GABA_A$ receptor function by $^{36}Cl^-$ influx, appear to be inconclusive, as well, with some studies showing that ethanol potentiates $GABA_A$ receptor function (3,29), while others, including the present one (Fig. 5), being unable to observe an effect of ethanol on $GABA_A$ receptor function (27,28).

The results from the electrophysiological and biochemical studies suggest that the interaction of ethanol with the $GABA_A$ receptor may be complex. Ethanol enhancement of $GABA_A$ receptor function depends on the subunit composition (7,35) and phosphorylation state of the receptor (12,35). It may also depend on the levels of some unknown factor (13,26) or the simultaneous activation of both $GABA_A$ and $GABA_B$ receptors (2), or some combination of the above.

The results from the present studies, demonstrating no differences in agonist-stimulated $^{36}Cl^-$ influx between P and NP rats, are in agreement with previous studies, which indicated no clear relationship between sensitivity to the acute intoxi-

cating effects of ethanol and agonist-stimulated $^{36}Cl^-$ influx into microsacs (3,6). The finding that pentobarbital enhances agonist-stimulated $^{36}Cl^-$ influx to the same extent in P and NP rats is also in agreement with previous studies using LS and SS mice (3). However, the fact that P and NP rats did not show any clear differences in the ability of phenobarbital (Figs. 3 and 4) or flunitrazepam (34) to enhance GABA-stimulated $^{36}Cl^-$ influx are not in agreement with the findings in LS and SS mice (4,10) and HAS and LAS rats (5). LS mice, compared to SS mice, are not only more sensitive to the acute intoxicating effects of ethanol (24), but are also more sensitive to the motor incoordinating effects of benzodiazepines (22,25), and to the acute intoxicating effects of phenobarbital (23,25,31). It is possible that the differences between the LS and SS mice to flunitrazepam and phenobarbital enhancement of agonist-stimulated $^{36}Cl^-$ influx might be a reflection of the differences between the lines to the behavioral effects of these agents. The sensitivity of the P and NP rats to the motor incoordinating and sedative effects of benzodiazepines and barbiturates has not been studied, but based on the present findings, P and NP rats might be hypothesized to have similar responses to barbiturates and benzodiazepines. Indeed, although individually housed P rats show greater levels of anxiety-like behavior compared to NP rats, the benzodiazepine, chlordiazepoxide, has equivalent anxiolytic-like activity in both P and NP rats (33).

In summary, under the present assay conditions, ethanol does not appear to potentiate GABA-stimulated Cl^- influx into cortical microsacs prepared from the P/NP and HAD/LAD lines of rats. Therefore, it is not possible to make any conclusions concerning interline differences in the response of the $GABA_A$ receptor to ethanol. However, in other measures of $GABA_A$/benzodiazepine receptor function examined, no obvious differences were found between the P and NP rats. The lack of an ethanol effect may be a result of subtle variations in the assay conditions or the loss of some important factor(s). It is also possible that ethanol may potentiate GABA-mediated events in other brain regions not examined or areas too small to measure with this technique, but that may be more intimately associated with differences in sensitivity to the acute intoxicating effects of ethanol than the cerebral cortex.

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