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Acute and Chronic Effects of Ethanol on Receptor-Mediated Phosphatidylinositol 4,5-Bisphosphate Breakdown in Mouse Brain

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SUMMARY

Phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown was stimulated by agonists acting at muscarinic cholinergic and α_1-adrenergic receptors in mouse brain. Ethanol, *in vitro*, inhibited basal cerebral cortical PIP_2 breakdown with a threshold concentration of 75–100 mM. Basal PIP_2 breakdown in hippocampus and striatum was less sensitive to ethanol. A high concentration of ethanol (500 mM) increased the EC_{50} for carbachol stimulation of PIP_2 breakdown in all three brain areas, but had no effect on the EC_{50} for norepinephrine. Following chronic ingestion of ethanol by mice, the EC_{50} for carbachol stimulation of PIP_2 breakdown in cortex was *decreased*, and there was no change in striatum. These effects were consistent with previously observed increases in quinuclidinylbenzilate (QNB) binding in cortex, but not striatum, of mice fed ethanol chronically. However, in hippocampus, where chronic ethanol ingestion had also induced an increase in QNB binding, the EC_{50} for carbachol stimulation of PIP_2 breakdown was *increased*. Binding studies using the specific M_1 muscarinic cholinergic receptor antagonist, pirenzepine, revealed that the number of pirenzepine-binding sites was increased in cortex, but not hippocampus (or striatum) of ethanol-fed mice. These results support the hypothesis that high affinity pirenzepine-binding sites are coupled to PIP_2 breakdown in mouse cortex. The changes in cerebral cortex represent one of the first demonstrations of a functional correlate of a change in receptor density in ethanol-treated animals. Increased sensitivity to cholinergic agonists in cortex may contribute to particular signs of ethanol withdrawal.

Ethanol has been found to modulate the coupling between neurotransmitter receptors and effectors which generate intracellular second messengers, e.g., AC (1–3). This action of ethanol was suggested to be mediated, in part, via its neuronal membrane-perturbing properties (1, 2, 4, 5). However, there are specific sites at which ethanol modifies the protein-protein interactions involved in receptor-AC coupling, and these sites vary in different brain areas (1–3, 6). Therefore, although the actions of ethanol may result from "fluidization" of membrane lipids (4, 5), the activities of particular membrane-bound proteins are affected in rather specific ways by ethanol.

Certain neurotransmitter receptors in the central nervous system are coupled to the catabolism of inositol lipids in the cell membrane (7–10). Occupation of these receptors by agonists results in the hydrolysis of PIP_2 by phospholipase C, to yield diacyglycerol and inositol triphosphate(s) (inositol 1,4,5-triphosphate and/or inositol 1,3,4-triphosphate) (7). The inositol triphosphates are then further metabolized by hydrolysis of the phosphate groups (7). Inositol triphosphate is believed to be a "second messenger" for calcium mobilization, and diacylglycerol can stimulate the calcium-dependent protein kinase C (7, 11). It has been suggested that agonists, by interacting with membrane-bound receptors, may alter the conformation of inositol lipids within the membrane, making them more accessible to phospholipase C (12). Recently, evidence has also been provided for a role of guanine nucleotide-binding proteins (N) in receptor-mediated stimulation of inositol lipid metabolism (7, 13). Ethanol might be expected to alter the activity of these systems, by perturbing membrane lipid properties and/or by interacting with N, as it does in the receptor-coupled AC systems (1–3, 6).

In the central nervous system, two of the receptors which are coupled to PIP_2 breakdown are the α_1-adrenergic receptor and the muscarinic cholinergic receptor (8–10), which appears to have two subtypes, designated M_1 and M_2 (14, 15). Based on interactions with the selective M_1 antagonist, pirenzepine, the receptors in cerebral cortex, striatum, and hippocampus have been postulated to be primarily the M_1 subtype (16–19), and have been proposed to be coupled to PIP_2 breakdown (9, 10,

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ABBREVIATIONS: AC, adenylate cyclase; PIP_2, phosphatidylinositol 4,5-bisphosphate; N, guanine nucleotide-binding protein; QNB, quinuclidinylbenzilate; CB, carbachol; EDTA, ethylene diaminetetraacetic acid; NE, norepinephrine.

17). In other tissues, the relationship between cholinergic receptor subtypes and PIP_2 breakdown is not as clear (20).

We (21) and others (22) have previously shown that, after chronic treatment of mice with ethanol, there was an increased number of muscarinic cholinergic receptor sites in cerebral cortex and hippocampus, but not striatum. These sites were quantitated using ^{3}H-QNB, which does not distinguish M_1 or M_2 receptor subtypes. The time course of appearance and disappearance of the changes in receptor density suggested that these changes might contribute to the development or expression of particular signs of ethanol withdrawal (21). However, in order to relate changes in receptor number to physiological symptoms of ethanol withdrawal, it is necessary to ascertain the functional relationship between the changes in receptor density and biochemical or physiological events. Since the muscarinic cholinergic receptors in the brain areas of interest are thought to be M_1, we investigated the acute (*in vitro*) and chronic (*in vivo*) effects of ethanol on receptor-stimulated PIP_2 breakdown in brain.

Methods

Chronic ethanol treatment. Male C57BL mice (22–25 g) were obtained from the National Cancer Institute (Frederick, MD) and were housed six per cage in our laboratories (12-hr light-dark cycle; 25°) for at least 1 week before use. For chronic ethanol treatment, mice were fed a liquid diet containing ethanol or were pair-fed a control liquid diet, for 7 days, by a method described previously (23). At the end of this period, all mice were given the control diet (withdrawal), and animals were sacrificed at this time for determination of PIP_2 breakdown. Tissue from control and ethanol-fed animals was prepared and assayed simultaneously in each chronic experiment. In most experiments, several other animals were monitored for withdrawal symptomatology (23). At 24 hr after withdrawal, when ethanol had been eliminated and overt withdrawal symptoms had subsided, these animals were also tested for tolerance to the hypnotic effect of a challenge dose of ethanol (3 g/kg) (23).

Determination of PIP_2 Breakdown. Animals were killed by decapitation and brains were rapidly removed and dissected on a chilled glass plate (21). Tissue was prepared and PIP_2 breakdown was assayed, using modifications of previously described methods (8–10, 24). Cross-chopped slices (350 μm) were cut with a McIlwain tissue chopper. For experiments with cortex, slices from 6–8 animals were pooled; for hippocampus, slices from 8–10 animals were pooled; and for striatum, slices from 15–20 animals were pooled. The slices were transferred to flasks containing 60 ml of modified Krebs-Henseleit buffer, pH 7.3–7.4 (10). The buffer was changed once, and the slices were gently agitated at 37° in a shaking water bath under an atmosphere of 95% O_2/5% CO_2 for 30 min. Buffer was then removed and the slices were transferred to a 15-ml conical centrifuge tube and allowed to settle ("packed slices").

The packed slices were incubated with 0.3 μM ^{3}H-*myo*-inositol (New England Nuclear, Boston, MA; 16.5 Ci/mmol) for 1 hr at 37°, with gentle shaking under an atmosphere of 95% O_2/5%CO_2. (The ^{3}H-*myo*-inositol was purified prior to use by passage through a column containing 1 ml of Bio-Rad AG 1x8, 200–400 mesh, formate form.) Following the incorporation step, the slices were washed four times with 40 ml of Krebs-Henseleit buffer at room temperature. Rates of basal and stimulated (CB, 10^{-4} M) PIP_2 breakdown were linear up to a volume of 40 μl of packed cortical slices, corresponding to a protein concentration (25) of about 2.0 mg/assay tube. Protein concentration was proportional to the volume of packed slices, and, for subsequent experiments, a volume of 30 μl of packed slices was chosen (approximately 1.4 mg of protein). The packed slices were added to assay tubes containing Krebs-Henseleit buffer, LiCl (10 mM), and agonists and/or antagonists at varying concentrations, in the presence or absence of alcohols. Total volume was 300 μl. Blank assay tubes contained slices that had incorporated ^{3}H-*myo*-inositol; these mixtures were placed in a boiling water bath for 10 min. Assays were carried out at 37° for 1 hr (based on preliminary findings which indicated constant reaction rates during this period), in capped tubes, with gentle shaking, in an atmosphere of 95% O_2/5% CO_2.

The reaction was stopped by addition of 1 ml of a CCl_3/MeOH (1:2) solution. Tubes were vortexed for 1 min, and 10 min later 0.35 ml of CCl_3 and 0.35 ml of H_2O were added to each tube. The solutions were mixed and centrifuged at 500 × *g* for 10 min at 10° in a Beckman J2-21M centrifuge. An aliquot of the organic layer (0.2 ml) was dried in a scintillation vial, and scintillation cocktail (3a70B, RPI Corp., Mt. Prospect, IL) was added. Radioactivity in the organic phase was quantitated on an LKB Rackbeta III liquid scintillation spectrometer (efficiency, 46%), for estimation of ^{3}H-*myo*-inositol incorporated into phospholipids. An aliquot (1.0 ml) of the aqueous phase was applied to a column containing 1.0 ml of Bio-Rad AG 1x8, 200–400 mesh, in the formate form. The column was washed twice with 10 ml of a solution of 250 mM *myo*-inositol, and once with 10 ml of a solution of 5 mM *myo*-inositol, and inositol phosphates were then eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid. Two-ml aliquots of the eluate were added to scintillation fluid, and radioactivity was quantitated as described above (efficiency, 35%). In a preliminary experiment, we determined that ethanol did not interfere with the extraction procedure.

For determination of results, total dpm in the organic and aqueous layers were calculated. Blanks were found to vary linearly with the amount of ^{3}H-*myo*-inositol incorporated into phospholipid, and each sample was corrected for its blank value based on this incorporation. Results were expressed as the ratio of dpm (minus blank) in the aqueous phase to the sum of dpm (minus blank) in the aqueous phase plus dpm in the organic phase (9). This method of expressing the results minimized variability due to differing amounts of slices in each assay tube, as well as differing degrees of incorporation in various experiments. The dose of agonist that produced half-maximal stimulation (EC_{50}) was determined from log-probit analysis of the data.

Pirenzepine binding. Brain tissue was dissected as described above, immediately frozen on dry ice, and stored at −70° until used for binding assays. Binding was assayed on cortical tissue from individual animals and on pooled hippocampal or striatal tissue from three animals, prepared as previously described (18), except that the buffer used was 10 mM Tris containing 1 mM EDTA and 0.9% NaCl, pH 7.4. ^{3}H-Pirenzepine binding was assayed as described previously (18). Scatchard plots (26) of binding data were used to estimate K_D and B_{max} values for pirenzepine binding.

Statistical analysis. Results were analyzed using analysis of variance, Student's *t*-test, or the paired *t* test (27), and the level of significance chosen was $p < 0.05$. The paired *t* test was used to compare values obtained when tissue from control and ethanol-treated animals was prepared and assayed simultaneously.

Reagents. CB and *myo*-inositol were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grade commercially available. Pirenzepine was a generous gift from Dr. R. Hammer (Thomae, Biberach an der Riss, Germany) and prazosin was kindly supplied by Pfizer Co., Ltd.

Results

NE and CB stimulated PIP_2 breakdown in cerebral cortex and hippocampus, and CB stimulated inositol lipid turnover in striatum, in a dose-dependent manner (Fig. 1). CB was more potent in stimulating PIP_2 breakdown in hippocampus and striatum than in cortex (Fig. 1), as indicated by the EC_{50} values for carbachol shown in Table 1. In contrast, the dose-response curves for NE (Fig. 1) and EC_{50} values for this neurotransmitter were similar in cortex and hippocampus (cortex, $EC_{50} = 3.0 \pm 0.3$ μM; hippocampus, $EC_{50} = 4.3 \pm 1.3$ μM, mean ± SE, $n = 3$ experiments). The dose-response curves for NE were steeper than those for CB, and Hill coefficients generated from these

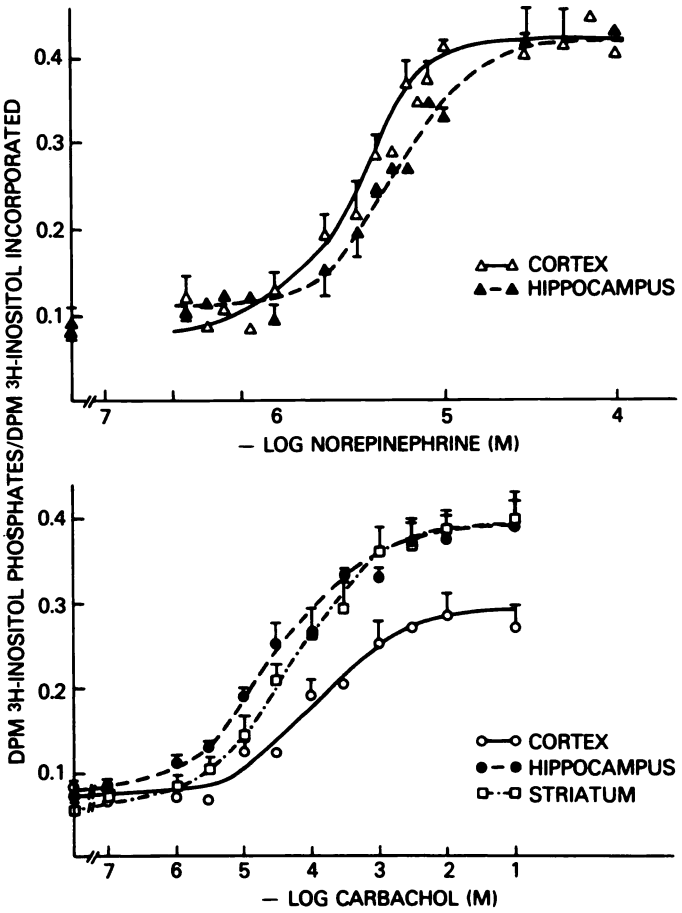

Fig. 1. Neurotransmitter-stimulated PIP_2 breakdown in brains of C57BL mice. PIP_2 breakdown was measured as accumulation of inositol phosphates in slices of mouse cortex, hippocampus, or striatum, as described in the text. Values represent means ± standard error from four experiments with each brain area.

TABLE 1

Effect of in vitro ethanol on CB-stimulated PIP_2 breakdown in mouse brain

CB-stimulated PIP_2 breakdown in slices of each brain area was determined as described in the text. EC_{50} is the concentration of CB that gave half-maximal stimulation of activity. Basal values in the absence of ethanol (reported as dpm of ^{3}H-inositol phosphates/dpm of ^{3}H-inositol incorporated, mean ± SE) were: cerebral cortex, 0.088 ± 0.008 (n = 16); hippocampus, 0.089 ± 0.007 (n = 11); striatum, 0.056 ± 0.014 (n = 3). Values in the presence of ethanol represent the mean ± standard error of three to six experiments; control and basal values are pooled from all experiments using a given brain area.

Additions to assay	EC_{50} (μM)		
	Cerebral cortex	Hippocampus	Striatum
None (control)	82.8 ± 9.8	33.1 ± 7.6[a]	47.0 ± 8.7[a]
Ethanol (50 mM)	86.4 ± 20.6	34.0 ± 15.4	28.9 ± 3.9
Ethanol (500 mM)	155.5 ± 17.9[b]	81.6 ± 19.3[b]	88.2 ± 23.1[b]
1-Butanol (50 mM)	162.5 ± 35.3[b]		

[a] $p < 0.05$ compared to cerebral cortex (analysis of variance and multiple t test).
[b] $p < 0.05$ compared to respective control [Student's t test, or analysis of variance and multiple t test (cortex)].

curves were >1. The stimulation of PIP_2 breakdown by NE was inhibited by the α_1-adrenergic receptor antagonist prazosin, whereas stimulation by CB was blocked by the M_1 muscarinic cholinergic receptor antagonist, pirenzepine (Table 2). These receptor antagonists did not significantly affect "basal" (unstimulated PIP_2 breakdown (Table 2).

Ethanol, added *in vitro* to the assays, affected both basal and

TABLE 2

Effect of receptor antagonists on CB- and NE-stimulated PIP_2 breakdown in mouse brain

PIP_2 breakdown in brain slices was measured as described in the text. Values represent the mean ± standard error from three experiments, or the mean from two experiments (range in parentheses). Activity is reported as dpm of ^{3}H-inositol phosphates/dpm of ^{3}H-inositol incorporated (see text). Prazosin was not tested in hippocampus. The concentration of CB used to evaluate the IC_{50} values for pirenzepine was 80 μM. IC_{50} values were obtained from log-probit plots of the data.

	PIP_2 breakdown	
	Cortex	Hippocampus
Muscarinic receptor		
Basal activity	0.068 ± 0.017	0.051 ± 0.019
Basal activity plus 10^{-5} M pirenzepine	0.075 ± 0.034	0.057 ± 0.018
Pirenzepine IC_{50} (nM) vs. CB-stimulated activity	164 (155–173)	377 (363–391)
Adrenergic receptor		
Basal activity	0.088 ± 0.009	
Basal activity plus 10^{-7} M prazosin	0.093 ± 0.004	
NE (3×10^{-6} M)	0.175 ± 0.029[a]	
NE plus prazosin	0.101 ± 0.005	

[a] $p < 0.05$ compared to basal activity.

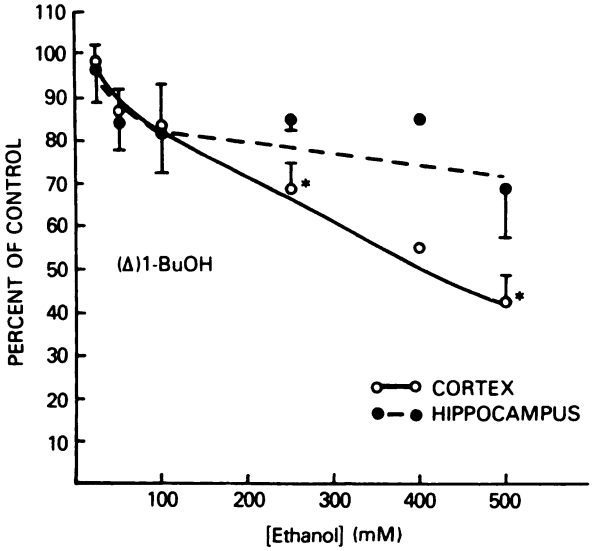

Fig. 2. Inhibition of basal PIP_2 breakdown by ethanol added *in vitro*. PIP_2 breakdown was measured as accumulation of inositol phosphates in slices of mouse cortex or hippocampus, as described in the text. Ethanol at the indicated concentrations was added to the assay tubes after the preincubation with ^{3}H-*myo*-inositol. Values represent means ± standard error from four to nine experiments. In two experiments, 50 mM 1-butanol was added to assays using cortical slices. *, $p < 0.05$ compared to value in the absence of ethanol (Student's t test).

receptor-stimulated PIP_2 breakdown, and its effects varied depending on the brain area studied and the neurotransmitter tested. Ethanol inhibited basal PIP_2 breakdown in cortex and hippocampus (Fig. 2). The threshold ethanol concentration for inhibition in cortex was 75–100 mM, and PIP_2 breakdown in cortex was more sensitive to higher concentrations of ethanol than that in hippocampus (Fig. 2). The IC_{50} for ethanol inhibition in cortex was 360 mM, whereas, in hippocampus, the IC_{50} was greater than 500 mM, and significant inhibition occurred only at 400–500 mM ethanol (Fig. 2). In cortex, 50 mM 1-butanol inhibited basal activity by 46% (Fig. 2). Since 50 mM 1-butanol was more potent than 50 mM ethanol, the inhibition was not simply a result of changes in medium osmolarity.

Ethanol had very little effect on basal PIP_2 breakdown in striatum (about 7% inhibition in the presence of 500 mM ethanol).

Ethanol *in vitro* also altered the stimulation of PIP_2 breakdown by CB (Table 1); however, significant changes in the EC_{50} value for carbachol in cortex, hippocampus, and striatum were only apparent at a high concentration of ethanol (500 mM). The changes were similar in all three brain areas, where 500 mM ethanol produced a 2–3-fold increase in the EC_{50} value for CB (Table 1). Ethanol at a concentration of 50 mM had no significant effect on the EC_{50} value for CB, whereas 50 mM 1-butanol increased the EC_{50} value for CB in cortex to the same extent as 500 mM ethanol, again indicating that osmolarity changes did not account for effects on EC_{50} values (Table 1). In a series of experiments, an ethanol dose-response curve was determined in the presence of 100 μM CB, to assess the threshold concentration for ethanol inhibition of activity. In cortex and hippocampus, the threshold concentration was 250 mM (5–10% inhibition).

In contrast to the effect on CB-stimulated PIP_2 breakdown, ethanol (500 mM) had no significant effect on the dose-response curves for NE stimulation of PIP_2 breakdown in cortex (EC_{50}, 2.7 μM) or hippocampus (EC_{50}, 5.3 μM). In all of these experiments, ethanol did not significantly alter the absolute value of maximal stimulation of PIP_2 turnover produced by CB or NE, although basal activity was lowered by 500 mM ethanol, as indicated above.

Chronic treatment of animals with ethanol resulted in functional tolerance to and physical dependence on ethanol (23). The results obtained when PIP_2 breakdown was measured in the brains of the ethanol-fed animals are shown in Table 3. We did not observe any consistent differences in the amount of ^{3}H-*myo*-inositol taken up by tissue of ethanol-treated animals as compared to controls, although these values varied depending on the amount of slices per assay tube. Basal PIP_2 breakdown in all brain areas was also similar in control and ethanol-treated mice (Table 3), and there were no significant differences between basal activities in control (liquid diet-fed) animals and in ethanol-naive (chow-fed) animals (compare Table 3 and legend to Table 1). Furthermore, there were no significant differences between control and ethanol-treated animals in the maximal stimulation of PIP_2 breakdown by NE or CB in each brain area (data not shown).

Ethanol treatment did not change the EC_{50} value for NE in cortex (Table 3). However, ethanol ingestion did alter the EC_{50} values for stimulation of PIP_2 breakdown by CB. In cortex, the dose-response curve for CB stimulation of PIP_2 breakdown was shifted to the *left* in ethanol-fed animals, compared to controls. Thus, the EC_{50} value for CB in brains of ethanol-treated animals was significantly *decreased*, as compared to that of controls (Table 3). There was no significant difference in EC_{50} values between animals fed control liquid diet (Table 3) and untreated chow-fed animals (Table 1). In contrast to the results in cortex, the EC_{50} value for CB was significantly *increased* in hippocampus of ethanol-treated animals, as compared to controls (Table 3) or untreated chow-fed animals (Table 1). In striatum, there was no statistically significant difference in EC_{50} values for CB among ethanol-treated mice, control mice, or untreated (chow-fed) mice (Tables 1 and 3).

The results of the pirenzepine binding studies are shown in Fig. 3. There was no significant difference in affinity for pirenzepine in any brain area of ethanol-fed animals as compared to controls. However, receptor density was significantly increased in cortex of ethanol-treated animals (20% increase). In contrast, there was no significant change in pirenzepine binding in hippocampus of ethanol-treated animals as compared to controls. There was also no significant change in striatal pirenzepine binding in ethanol-fed mice. In the present studies, changes in QNB binding (performed as described in Ref. 18) were very similar to those reported earlier (21, 22) (data not shown).

TABLE 3

Effect of chronic in vivo ethanol treatment on receptor-mediated PIP_2 breakdown in mouse brain

Receptor-mediated PIP_2 breakdown was measured as described in the text in control mice or mice that had ingested ethanol for 7 days. Mice were sacrificed at the time of withdrawal. Basal activity is reported as dpm of ^{3}H-inositol phosphates/dpm of ^{3}H-inositol incorporated. EC_{50} values are the concentrations that gave half-maximal activation. Values represent mean ± standard error or means of two experiments where the values did not differ by more than 10% from the mean.

	n[a]	Control	Ethanol-treated
Cortex			
Basal activity	8	0.090 ± 0.010	0.077 ± 0.010
CB EC_{50} (μM)	6	107.9 ± 15.1	62.7 ± 13.7[b]
NE EC_{50} (μM)	2	3.7	4.1
Hippocampus			
Basal activity	4	0.062 ± 0.010	0.065 ± 0.012
CB EC_{50} (μM)	4	46.5 ± 13.4	78.7 ± 14.5[b]
Striatum			
Basal activity	3	0.056 ± 0.014	0.079 ± 0.032
CB EC_{50} (μM)	3	22.5 ± 6.2	23.4 ± 6.7

[a] *n*, number of experiments.
[b] $p < 0.05$ compared to control (paired *t* test).

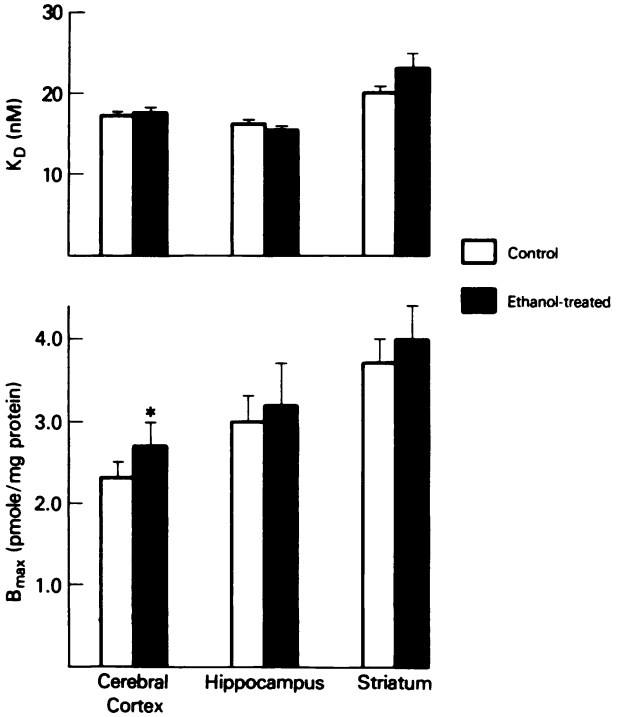

Fig. 3. Pirenzepine binding in brains of mice treated chronically with ethanol. ^{3}H-Pirenzepine binding was assayed as described in the text in tissue of control mice or mice that had ingested ethanol in a liquid diet for 7 days. Mice were sacrificed at the time of withdrawal from ethanol. Values represent means ± standard error of nine (cortex) or three (hippocampus and striatum) experiments for each group. *, $p < 0.05$, compared to control.

Discussion

The characteristics of stimulation of PIP_2 breakdown by CB and NE in cerebral cortex and hippocampus of the C57BL mouse were similar to those reported for the same areas of rat brain (8–10), although we found no indication of a biphasic NE dose-response curve, as was reported for rat hippocampus (8). The difference in potency for CB between mouse striatum and cortex was qualitatively similar to that reported for these areas of guinea pig brain (28).

The effects of ethanol, added *in vitro*, on PIP_2 breakdown were rather selective for brain area and neurotransmitter. The threshold ethanol concentration for inhibition of basal PIP_2 breakdown in cortex, 75–100 mM, is in the range that can be attained *in vivo* (23, 29). Thus, activity in this brain area, in contrast to hippocampus or striatum, could be altered during ethanol ingestion. Basal activity in cortex was not affected by prazosin or pirenzepine and is therefore apparently not dependent on endogenous agonist interaction with neurotransmitter receptors. This finding suggests that the effect of ethanol on basal activity probably occurs distal to the receptors. Presumably, basal PIP_2 breakdown reflects the tonic activity of phospholipase C, which hydrolyzes PIP_2 (12). However, general inhibition of enzyme activity would not account for the selective sensitivity to ethanol in different brain areas. It is possible, as postulated for agonists (12), that ethanol affects the conformation of the substrate through its lipid-perturbing properties (4, 5). Differences in the lipid composition of neuronal membranes in different brain areas could then contribute to the differential sensitivity to ethanol.

The effects of ethanol on agonist-stimulated PIP_2 breakdown were also selective, but the differences were related to the receptor being examined, rather than to brain area. The ethanol-induced shift in the dose-response relationship for CB stimulation of PIP_2 breakdown may account for results of an earlier investigation, in which 100 mM ethanol reduced CB (10^{-3} M)-stimulated incorporation of ^{32}P into phosphatidic acid in synaptosomes from mouse forebrain (30). In the present study, the fact that ethanol did not alter NE stimulation of PIP_2 breakdown in cortex or hippocampus indicates that ethanol did not simply have a nonspecific toxic effect on the brain slice preparations, but selectively affected the function of the muscarinic cholinergic receptors.

Ethanol could alter the EC_{50} for carbachol by interfering with ligand binding to the muscarinic cholinergic receptor. However, our prior data (21) showed no effect of low doses of ethanol on QNB binding in mouse brain, and no reports of effects of higher concentrations are available. If ethanol does not interfere with agonist binding to brain cholinergic receptors, then some aspect of the coupling between the muscarinic cholinergic receptor and PIP_2 breakdown may be affected by ethanol. In systems where brain receptors are coupled to AC, ethanol primarily altered the function of the guanine nucleotide-binding protein (1–3, 6). Considering the evidence for a role for such a protein in receptor-stimulated PIP_2 breakdown (7, 13), a selective effect of ethanol on this coupling process also seems possible. The fact that a high concentration of ethanol was necessary to produce a significant effect suggests that the lipid-perturbing properties of ethanol may be involved in this action (4, 5, 31). The comparative potencies of butanol and ethanol also support the hypothesis that the effects of the alcohols may be related to their lipid solubility and, possibly, their membrane-perturbing effects.

In the present studies, we did not separate the individual inositol phosphates released from PIP_2, and it seems likely that the major product which accumulated, particularly after CB stimulation, was inositol-1-phosphate, as was reported for rat brain (9). However, a recent study suggests that inositol mono- and diphosphates may not be derived only from inositol triphosphate in rat cortex (32). Although a change in the proportion of various metabolites released would not alter the overall conclusions of the present studies, such considerations might be important for further elucidating the mechanism of action of ethanol.

The *decrease* in EC_{50}—i.e., increase in potency—for CB to stimulate PIP_2 breakdown, which was seen in cortex of mice that had ingested ethanol chronically, is characteristic of systems in which there is an increase in receptor number (33). Increases in QNB binding had been found in cortex of mice after chronic ethanol ingestion (21, 22). In striatum, where no increase in QNB binding was found after chronic ethanol treatment (21), there was no change in the potency of CB to stimulate PIP_2 breakdown. These results represent one of the first demonstrations of a functional, biochemical correlate to a change in neurotransmitter receptor number in ethanol-treated animals. Increased sensitivity to cholinergic agonists in cortex could well play a role in the development or expression of ethanol withdrawal symptomatology. The increased sensitivity to CB in cortex could also represent an adaptive response to the acute depressant effect of ethanol on basal PIP_2 breakdown.

The correlation between change in receptor number and change in the response to CB did not hold in the hippocampus, however. In this brain area, the potency of CB was *decreased* after chronic ethanol treatment, whereas QNB binding studies demonstrated an increase in the number of binding sites (21). Thus, the hippocampal system appeared to undergo a pathological, rather than an adaptive change.

Based on studies of pirenzepine inhibition of cholinergic agonist-stimulated inositol lipid metabolism, it was suggested that the same cholinergic receptor subtype (possibly M_1) was coupled to PIP_2 breakdown in guinea pig cortex and hippocampus (28). In our studies, the estimated K_i values for pirenzepine inhibition of CB-stimulated PIP_2 breakdown were 82 nM[1] in mouse cortex and 110 nM in mouse hippocampus. These values are intermediate between the K_D values for pirenzepine binding to high affinity sites (M_1) (9–20 nM) (Ref. 18; Fig. 3) and low affinity sites (M_2) (190–600 nM) (18, 28) in brain. The similarity of the K_i values in cortex and hippocampus, however, suggests that, as in guinea pig brain (28), the same receptor subtype may be coupled to inositol lipid metabolism in both mouse brain regions. It must be pointed out that binding studies and measurements of PIP_2 breakdown were carried out under very different conditions, making comparisons of the K_i and K_D values difficult. However, in mouse cortex, both the density of high affinity (K_D = 17 nM) pirenzepine-binding sites and the potency of CB to stimulate PIP_2 breakdown were increased after chronic ethanol treatment. This correlation may provide a better indication that the high affinity pirenzepine-binding site is coupled to PIP_2 breakdown in cortex. The data in

[1] The K_i value was estimated by dividing the IC_{50} value by 1 plus the concentration of carbachol divided by the EC_{50} for carbachol (34).

hippocampus were not as clear, but the converse correlation—i.e., high affinity pirenzepine binding was *not* increased, and the EC_{50} for CB stimulation of PIP_2 breakdown was *not* decreased after chronic ethanol treatment—supports the hypothesis that the M_1 receptor subtype may also be coupled to PIP_2 breakdown in hippocampus. The previously noted increase in QNB-binding sites in hippocampus of ethanol-fed mice (21) apparently represents a subtype of muscarinic cholinergic receptor that is not coupled to PIP_2 breakdown.

The results presented illustrate the selective nature of the responses of various receptor systems coupled to PIP_2 breakdown to the acute and chronic effects of ethanol and also emphasize the importance of evaluating functional responses to agonists in addition to measuring receptor binding. The attempt to correlate changes in receptor number with functional consequences of receptor activation is necessary in order to understand the biochemical basis for adaptive or maladaptive behavioral responses which occur in individuals who are tolerant to and physically dependent on ethanol.

Acknowledgments

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