Biochemical Pharmacology, Vol. 33, No. 22, pp. 3591–3595, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

BRAIN MEMBRANE DISORDERING AFTER ACUTE *IN VIVO* ADMINISTRATION OF ETHANOL, ISOPROPANOL OR *t*-BUTANOL IN RATS

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(*Received* 10 *February* 1984; *accepted* 28 *May* 1984)

Abstract—Brain synaptosomal membranes were prepared from rats sacrificed 18 hr after a single intragastric dose of water or of ethanol (100 mmol/kg), when blood ethanol had fallen almost to zero. Fluorescence polarization of DPH, and $(Na^+ + K^+)$ATPase activity, were studied in these membranes in the presence of 0, 0.175, 0.3 or 0.7 M ethanol *in vitro*. After *in vivo* ethanol, basal ATPase activity was slightly increased, membrane fluidity was unchanged, but both measures showed increased sensitivity to the effects of ethanol *in vitro*. Similar results were found after an equivalent *in vivo* dose of isopropanol, but not of *t*-butanol. These findings indicate that the sensitization to *in vitro* effects of ethanol or isopropanol, after *in vivo* treatment with these alcohols, is probably not dependent principally on their lipid solubilities.

There are a number of reports providing evidence that if ethanol acutely increases fluidity in synaptosomal membranes, then chronically, it decreases it [1, 2]. Actually, after chronic treatment most investigators have found no change in baseline fluidity but a decreased susceptibility to fluidization by addition of ethanol *in vitro* [3, 4]. In contrast to this finding concerning chronic ethanol administration, we showed recently [5] that after a single *in vivo* ethanol administration to a naive animal the sensitivity of crude synaptic membranes to ethanol added *in vitro* is clearly enhanced. This hypersensitization could be caused by a physical effect of ethanol, such as its ability to disorder membrane lipids [6], and disappeared with further ethanol administration.

If the 'membrane disordering' hypothesis is correct, then other short-chain alcohols with high lipid solubility [7, 8] should produce a similar phenomenon. To test this possibility, the effects of administration of ethanol, isopropanol or *t*-butanol on synaptosomal membrane ordering were quantified in the present work using fluorescence polarization, carried out with DPH as a probe of the membrane lipid environment [9] and measurement of the activity of $(Na^+ + K^+)$ATPase, an enzyme which is integrated in the neuronal membranes and altered by changes in membrane characteristics [10]. Fluidity and ATPase activity were evaluated after adding different alcohol concentrations to the membrane preparations.

Our results show that the increase in basal synaptosomal $(Na^+ + K^+)$ATPase activity as well as the hypersensitivity found after *in vivo* acute ethanol treatment are also apparent and even more marked after *in vivo* administration of isopropanol, an amphiphilic molecule. However such changes are not found after *in vivo* administration of *t*-butanol, a more lipophilic alcohol.

MATERIALS AND METHODS

Alcohol administration. Male Sprague Dawley rats (Elevage Janvier, 53680 Le Genest, France) weighing 150–200 g were housed individually and fed with a standard laboratory chow (UAR, 91360 Villemoisson-sur-Orge, France) with free access to water.

The different alcohols were administered by gastric tube as a 20% v/v solution in water. Taking their lipid solubilities and their metabolic rates into consideration, membrane disordering was studied 18 hr after administration of ethanol at the dosage of 100 mmol/kg bwt, 20 hr after isopropanol (50 mmol/kg bwt), 40 hr after *t*-butanol (25 mmol/kg) and 24 hr after *t*-butanol (12.5 mmol/kg) [11–13]. An equal volume of water was administered to the corresponding control rats. Blood alcohol levels were determined by gas–liquid chromatography as previously described [11, 12].

Preparation of crude synaptosomal membranes. Animals were killed by decapitation, and the whole brain was quickly removed and homogenized in ten volumes of ice-cold 0.32 M sucrose containing 5 mM Tris–HCl buffered at pH 7.4. The crude P_2 fraction was then prepared and lysed as previously described [4, 5]. The final pellet (P_2M) enriched with synaptic membranes was resuspended in pH 7.4 phosphate buffer and kept at 4° before analysis [14]. The protein concentration (about 10–15 mg/ml) was determined after Lowry *et al.* [15].

Fluorescence probe polarization. An aliquot (1 ml) of the crude synaptosomal fraction (containing about 15 μg protein) was incubated at 37° for 60 min with 1 nmole of 1,6-diphenyl-1,3,5-hexatriene (DPH) dissolved in 1 μl tetrahydrofuran. Fluorescence polarization was then recorded at 25° using a SLM 8000 spectrophotofluorimeter (Urbana, U.S.A.) (with a

MC 320 monochromator and Glan Thomson polarizers), temperature in the cuvette being maintained at 25° by a thermistor. (Measurements were performed at 25° rather than at 37° in order to minimize the scattering of the data due to increasing motion of the probe with temperature. Nevertheless, control experiments, not reported here, were carried out at 37° to ensure the results and the comparison with ATPase findings.) The excitation wavelength was 360 nm, the emission being detected at 430 nm. The degree of fluorescence polarization P was calculated after Shinitzky and Barenholz [16]. After the determination of the basal values, a desired concentration of alcohol was added to the cuvette and P was again determined when polarization had reached a new steady state. Five determinations were averaged at each ethanol concentration for each different membrane preparation. In any case, addition of alcohol gave similar results at 37° and 25°.

$(Na^+ + K^+)$ATPase *assay*. $(Na^+ + K^+)$ATPase activity was obtained by subtracting the ouabain-insensitive activity from the total activity, and was expressed as micromoles of Pi produced per mg protein per hour. The assay mixture contained 30 mM imidazole, 30 mM glycylglycine, 3 mM $MgCl_2$, 3 mM Na_2ATP, 120 mM NaCl, 5 mM KCl, as well as the desired final concentration of alcohols and 0.05 ml of the diluted crude synaptosomal fraction (about 20 μg protein/ml) in a final volume of 1.3 ml at 37°C [17]. In another tube, 1 mM ouabain was added, and NaCl and KCl were omitted. The reaction was stopped after 20 min by addition of 0.5 ml of 1.2 M perchloric acid. Inorganic phosphate was determined with ammonium molybdate and 1-amino-2-naphthol-4-sulphonic acid [18].

Reagents. Na_2ATP (vanadium-free), ouabain, glycylglycine, imidazole, bovine serum albumin and reagents for blood-alcohol determination were from Sigma (St Louis, MO, U.S.A.), 1-amino-2-naphthol-4-sulphonic acid from BDH (Poole, Great Britain) and DPH from Aldrich-Europe (Beerse, Belgium). Purity of ethanol, isopropanol and *t*-butanol (Prolabo, Paris, France) was checked by gas chromatography. All other reagents were of analytical grade.

Expression of results. The experimental results are expressed as mean values ±S.E.M. Differences between the control and the alcohol-treated groups were analysed by Student's *t*-test.

RESULTS

(1) *Acute ethanol administration to naive animal*. Eighteen hours after the administration of an acute dosage of ethanol (100 mmol/kg bwt, p.o.) to four naive rats, blood alcohol was almost negligible.

No change in the basal synaptosomal fluidity was found whereas a statistically significant increase (+25%) in the activity of $(Na^+ + K^+)$ATPase was observed in the ethanol-treated animals when compared to the controls (Table 1).

In crude synaptosomes from both ethanol-treated and control animals, the *in vitro* addition of 0.175–0.7 M ethanol produced an increase in fluidity as well as an inhibition of $(Na^+ + K^+)$ATPase activity in a concentration-dependent manner. However, as previously shown [5, 14], at every ethanol concentration used, the changes in fluidity and in $(Na^+ + K^+)$ATPase activity were significantly more marked in membranes prepared from the ethanol-intubated animals (Table 1).

(2) *Acute isopropanol administration to naive animals*. Blood isopropanol had almost disappeared at time of sacrifice, 20 hr after intubation (Table 2). As after ethanol administration, intrinsic fluidity was unchanged and basal $(Na^+ + K^+)$ATPase activity was slightly increased (+ 21%) in the membrane from isopropanol treated animals compared to controls (Table 2). *In vitro* addition of ethanol (0.175–0.7 M) produced changes in membrane fluidity and in $(Na^+ + K^+)$ATPase activity which are significantly larger in membranes prepared from isopropanol-intubated animals than from control rats (Table 2).

Table 1. Blood ethanol and brain membrane parameters 18 hr after administration of ethanol (100 mmol/kg, p.o.) to rats

	Animal treatment	
	Control (4)	Ethanol (4)
Blood ethanol (mM)	—	0.11 ± 0.04
Synaptosomal fluorescence polarization of DPH		
Basal value P	0.335 ± 0.002	0.336 ± 0.002
[$\Delta P \times 10^3$] after *in vitro* addition of ethanol		
at 0.350 M	4.4 ± 0.5	7.5 ± 0.3†
at 0.700 M	9.6 ± 0.9	17.0 ± 1.6†
Synaptosomal $(Na^+ + K^+)$ATPase		
Basal value (μmoles/mg protein/hr)	15.75 ± 1.01	19.71 ± 1.14*
Percentage/inhibition after *in vitro* addition of ethanol		
at 0.175 M	15.93 ± 3.28	25.65 ± 1.66*
at 0.300 M	27.59 ± 1.02	37.01 ± 2.29†
at 0.700 M	47.92 ± 1.60	59.64 ± 1.68†

The values are means ± S.E.M. with the number of animals in parentheses.
P values (ethanol vs control group): * $0.02 < P < 0.05$, † $P < 0.01$.

Table 2. Blood isopropanol and brain membrane parameters 20 hr after administration of isopropanol (50 mmol/kg, p.o.) to rats

	Animal treatment	
	Control (4)	Isopropanol (4)
Blood isopropanol (mM)	—	0.33 ± 0.21
Synaptosomal fluorescence polarization of DPH		
Basal value P	0.335 ± 0.001	0.334 ± 0.002
[$\Delta P \times 10^3$] after *in vitro* addition of ethanol		
at 0.350 M	6.0 ± 0.6	9.3 ± 0.3‡
at 0.700 M	10.0 ± 0.9	17.5 ± 1.8‡
Synaptosomal ($Na^+ + K^+$)ATPase		
Basal value (μmoles/mg protein/hr)	17.11 ± 1.58	20.66 ± 1.95*
Percent inhibition after *in vitro* addition of ethanol		
at 0.175 M	14.87 ± 2.30	28.40 ± 1.92‡
at 0.300 M	27.68 ± 2.82	38.07 ± 0.70†
at 0.700 M	47.74 ± 2.35	60.07 ± 1.93‡

The values are means ± S.E.M. with the number of animals in parentheses.
P values (isopropanol vs control group): * $0.02 < P < 0.05$, † $0.01 < P < 0.02$, ‡ $P < 0.01$.

It should be noted that, as after ethanol, because of the induced elevation of basal ATPase, the enhanced inhibition brought in fact the activity down to very similar final values in preparations from alcohol-treated as from control animals whereas the enhanced fluidization was more clear-cut.

(3) *Acute t-butanol administration to naive animals.* *t*-Butanol was almost cleared from the blood 40 hr after its administration at a dose of 25 mmoles/kg, p.o. (Table 3). At that time, no change was found in the basal synaptosomal fluidity or in the activity of ($Na^+ + K^+$)ATPase.

Furthermore, in contrast to what was found after ethanol and isopropanol administration, no significant differences in sensitivity to ethanol added *in vitro* were observed between the controls and the *t*-butanol-treated animals (Table 3). The same held true when *t*-butanol was administered at a dose of 12.5 mmol/kg, p.o., the determinations being done 24 hr later (Table 4).

(4) In vitro *relative membrane-disordering potency of the three alcohols studied.* The effect of each alcohol added at 250 mM (final concentration) on the fluorescence polarization of DPH was tested both on membranes from control animals and on membranes from animals which were pretreated with the same alcohol (Table 5). In membranes from control animals, changes in degree of fluorescence polarization were correlated to the membrane/buffer partition coefficients as published by McCreery and Hunt [6, 19]. That did not hold true in membranes from pretreated animals. *In vivo* pretreatment by isopropanol-enhanced isopropanol *in vitro* effects; in contrast, *in vivo* pretreatment by *t*-butanol either at 25 mmol/kg or at 12.5 mmol/kg did not affect the response to *in vitro* addition of ethanol (Tables 3 and 4) or *t*-butanol (Table 5).

Table 3. Blood *t*-butanol and brain membrane parameters 40 hr after administration of *t*-butanol (25 mmol/kg, p.o.) to rats

	Animal treatment	
	Control (6)	*t*-butanol (6)
Blood *t*-butanol (mM)	—	0.10 ± 0.05
Synaptosomal fluorescence polarization of DPH		
Basal value P	0.339 ± 0.001	0.339 ± 0.001
[$\Delta P \times 10^3$] after *in vitro* addition of ethanol		
at 0.350 M	5.4 ± 0.6	6.1 ± 0.8
at 0.700 M	10.0 ± 0.9	10.4 ± 0.6
Synaptosomal ($Na^+ + K^+$)ATPase		
Basal value (μmoles/mg protein/hr)	19.19 ± 1.12	19.92 ± 1.02
Percent inhibition after *in vitro* addition of ethanol		
at 0.175 M	22.52 ± 1.78	15.56 ± 3.36
at 0.300 M	30.18 ± 1.80	36.76 ± 2.45
at 0.700 M	50.76 ± 2.20	52.03 ± 2.79

Expression of the results as in Tables 1 and 2.

Table 4. Blood *t*-butanol and brain membrane parameters 24 hr after administration of *t*-butanol (12.5 mmol/kg, p.o.) to rats

	Animal treatment	
	Control (3)	*t*-Butanol (3)
Blood *t*-butanol (mM)	—	0.26 ± 0.15
Synaptosomal fluorescence polarization of DPH		
Basal value *P*	0.340 ± 0.002	0.339 ± 0.001
[$\Delta P \times 10^3$] after *in vitro* addition of ethanol		
at 0.350 M	6.0 ± 0.6	6.7 ± 0.6
at 0.700 M	11.2 ± 0.9	11.9 ± 0.3
Synaptosomal ($Na^+ + K^+$)ATPase		
Basal value (μmoles/mg protein/hr)	21.47 ± 2.33	20.25 ± 1.31
Percent inhibition after *in vitro* addition of ethanol		
at 0.175 M	19.07 ± 3.39	15.28 ± 1.82
at 0.300 M	30.22 ± 2.04	28.26 ± 2.23
at 0.700 M	53.81 ± 2.76	55.30 ± 1.74

Expression of the results as in Tables 1 and 2.

DISCUSSION

Membrane disordering seems to be an essential step in the progression of events leading to intoxication by ethanol, and to be involved in the development of tolerance and dependence [20]. The effect of a single ethanol administration has however not been extensively investigated. McQuarrie and Fingl [21] reported a transient state of CNS hyperexcitability as evidenced by transient lowering of experimental threshold to evoked seizures in mice after such a single exposure to ethanol in previously naive animals. They argued that acute excitatory effects might be related to a manifestation of physical dependence, representing a mini withdrawal syndrome. McComb and Goldstein [8] also reported that a mild withdrawal reaction was seen after a single injection of ethanol. This hyperexcitability could be linked to the hypersensitivity we found in synaptic membranes at a time when ethanol is almost cleared from the blood [5, 14].

These effects of ethanol are not unique among alcohols. Acute isopropanol administration also appeared to sensitize synaptic membranes to the disordering effect of ethanol or isopropanol as detected by the fluidizing effect as well as by the ($Na^+ + K^+$)ATPase inhibition after *in vitro* addition of ethanol or isopropanol. Nevertheless, *t*-butanol, an alcohol with a high lipid solubility, failed to sensitize the membranes. This result was first observed in the present study after an administration of 25 mmol/kg. This dosage resulted in a high concentration of several hours duration [12, 13], due to the slow rate of *t*-butanol metabolism [22]. As the length of membrane exposure to *t*-butanol could allow some adaptation or repairing of the membranes masking the withdrawal reaction, another experimental condition with a smaller *t*-butanol dose (12.5 mmol/kg) was also used, but gave the same negative result.

These findings concerning *t*-butanol were quite unexpected, as it was shown previously by McComb and Goldstein [8] that *t*-butanol produces physical dependence, being 4–5 times more potent than ethanol. It was furthermore shown in the same laboratory [7] that the disordering potency of a series of short-chain alcohols, including *t*-butanol, on brain membranes is closely related to their membrane lipid

Table 5. Changes in fluorescence polarization of DPH after *in vitro* addition of the different alcohols studies

In vivo pretreatment	*In vitro* addition (250 mM final concentration)	$\Delta P \times 10^3$
Control (3)	Ethanol	5.3 ± 0.6
Ethanol (100 mmol/kg, 18 hr) (3)	Ethanol	7.1 ± 0.9*
Control (4)	Isopropanol	9.0 ± 0.8
Isopropanol (50 mmol/kg, 20 hr) (4)	Isopropanol	14.5 ± 2.0*
Control (4)	*t*-butanol	12.5 ± 1.6
t-butanol (25 mmol/kg, 40 hr) (4)	*t*-butanol	14.9 ± 0.3
t-butanol (12.5 mmol/kg, 24 hr) (3)	*t*-butanol	11.9 ± 0.3

The values are means ± S.E.M. with the number of animals in parentheses.
P values (alcohol pretreated group vs control group): * $P < 0.05$.

solubility. In accordance with this result, our present data show that *t*-butanol added *in vitro* to synaptosomal preparations from naive rats produces changes in the fluorescence polarization of DPH which are more marked than the changes observed with either ethanol or isopropanol addition. The lack of hypersensitivity to the *in vitro* addition of ethanol or *t*-butanol itself in animals having received a single *t*-butanol injection seems therefore surprising.

This lack of hypersensitivity, which opposes clearly sets *t*-butanol apart from ethanol, might be related to the fact that a single alcohol injection to naive rats is followed by a mild withdrawal reaction in the case of ethanol but not of *t*-butanol [8]. Such a withdrawal syndrome resulting in adrenergic hyperactivity [23, 24] could well be responsible for the increase in the basal synaptosomal $(Na^+ + K^+)$ATPase activity found in ethanol but not in *t*-butanol-treated animals.

As we have previously shown, however that stress conditions do not induce synaptosomal membrane hypersensitivity [5, 14]. It seems unlikely that adrenergic hyperactivity could be involved in the differences of membrane sensitivity to the *in vitro* addition of ethanol in rats receiving *t*-butanol or ethanol, and also that the apparent sensitization to ATPase inhibition is not only a reversal of the withdrawal-induced elevation of the basal level.

The three alcohols studied give rise to amphiphilic or lipophilic metabolites and distinct effects of these products could be considered to explain the difference in the alcohol actions. However, such an interaction is not supported by examination of the metabolism of the alcohols. In the case of ethanol and *t*-butanol when blood alcohol concentration is about negligible, the same holds true for the metabolites:acetaldehyde for ethanol, formaldehyde or acetone for *t*-butanol [11–13]. Besides, the metabolites have been present all during the alcohol exposure at very low concentrations which do not interfere significantly with the membrane organization. In contrast, high levels of acetone are produced during isopropanol exposure and acetone is present in an important blood concentration 20 hr after alcohol administration [11]. Besides, it was found that acetone, a lipophilic agent, administered in conditions to give the same blood level as after isopropanol treatment, did not produce membrane sensitization. Thus, it seems unlikely that the phenomenon of hypersensitization found after ethanol or isopropanol administration is related to any effect of their metabolites.

In conclusion, a not yet identified mechanism, including, for example, changes in membrane protein conformation [25] or actions at the polar membrane surface level [26], might intervene in the fact that the lipid solubility of the alcohol administered to naive animals is not the main determinant of the sensitivity of the synaptosomal membranes of these animals to ethanol added *in vitro*.

Acknowledgements—We are deeply indebted to Dr A. Alfsen and Mr M. Vincent (INSERM U221) for their advice concerning fluorescence polarization measurements. This investigation was supported by grants from INSERM (CRL 82 70 10) and from IREB (83-11).

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