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Alterations in phospholipid methylation in rat brain synaptosomal membranes produced by ethanol *in vitro* and *in vivo*

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The effects of alcohols on the CNS are considered by many to depend on entry into the synaptic membrane lipid bilayer with a consequent change in the physical properties of the membrane [1, 2]. This physical change, often referred to as a 'fluidisation', may then disrupt the activity of the various membrane-associated proteins and lipids responsible for synaptic transmission. Among the proteins which are likely to be most affected by such an action are enzymes which are located within the membrane bilayer and which have as their substrates the lipids of the bilayer. The enzymes responsible for the progressive methylation of phosphatidylethanolamine to phosphatidylcholine are examples of this kind of enzyme [3, 4] and the fact that they appear to be coupled to receptor protein activation [5] and calcium transport [5] make them of considerable interest in relation to synaptic function.

Current concepts of the mechanism of development of tolerance to alcohols indicate that the fluidisation which is produced in synaptic membranes is reduced when these are taken from ethanol-tolerant animals [6] and that this is due to some alteration in the lipid composition of these membranes [7–9]. If this is so one would expect the activity of membrane-bound enzymes also to be changed in synaptic membranes from ethanol-tolerant animals. We have therefore undertaken investigations of the effects of ethanol *in vitro* on the activity of phospholipid-methylating enzymes in synaptosomal preparations of control rats, as well as those from rats made tolerant and physically dependent on ethanol.

Methods and results

Male Sprague–Dawley rats (200–250 g) obtained from Charles River U.K. were used in these experiments. Rats were killed by decapitation, and whole brain dissected in the cold and synaptosomes prepared by the method of Cotman [10]. Synaptosomes were then incubated either in 10 mM HEPES buffer (pH 7.4) containing 4 mM dithiothreitol (DTT), 6% sucrose, 5 mM $MgCl_2$, 40 μM $CaCl_2$, or in 'artificial CSF' [11] for 1 hr at 37° in the presence of 200 μM *S*-adenosyl-L-methionine (SAM) containing 1 μCi of *S*-adenosyl-L-([^{3}H]methyl)-methionine [15 Ci/mmole (Radiochemical Centre, Amersham, U.K.)] as the methyl donor. At the end of this period synaptosomal lipids were extracted with chloroform : methanol : HCl (2 : 1 : 0.02 v/v/v) followed by repeated washings with 0.1 M KCl in 50% methanol. Aliquots of the chloroform phase were then used for scintillation counting. Alternatively, lysed synaptosomes (vigorous shaking in distilled water for 1 hr at 4°) were subjected to filtration (0.45-μm Millipore filters) before being taken for scintillation counting. These meth-

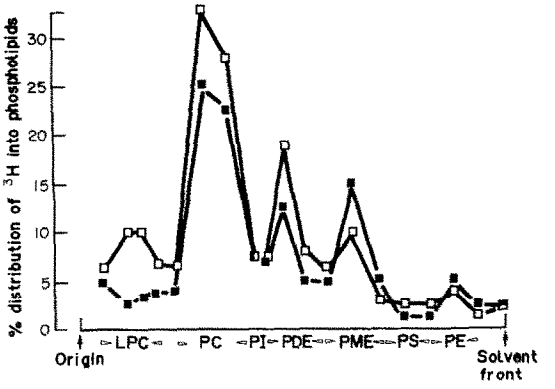

Fig. 1. Chromatographic pattern of percentage distribution of [^{3}H]methyl into phospholipids of rat brain synaptosomes in the presence (□) and absence (■) of 5×10^{-5} M isoprenaline. The incubation conditions are as described in the text, except that the amount of [^{3}H]SAM was increased to 4 μCi. The chromatograms were developed in a solvent system of chloroform:propionic acid:*n*-propyl alcohol:water (2:3:2:1 v/v/v/v) to a distance of 17 cm from the origin. Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PI, phosphatidylinositol; PDE, phosphatidyl-*N,N*-dimethylethanolamine; PME, phosphatidyl-*N*-monoethylethanolamine; PS, phosphatidylserine; PE, phosphatidylethanolamine.

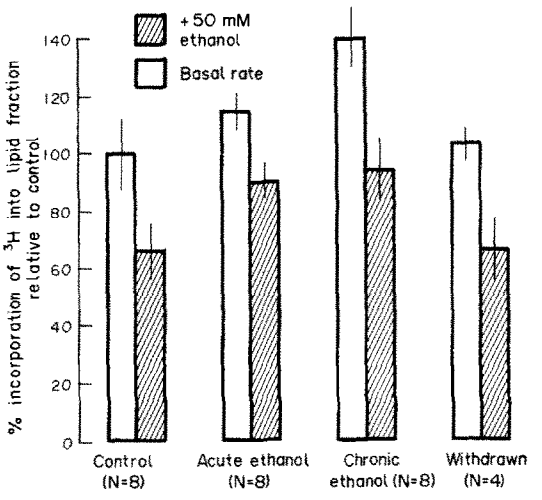

Fig. 3. Histograms of *in vitro* incorporation of ^{3}H into lipid fraction of rat brain synaptosomes after exposure to various ethanol treatments *in vivo*. The assays were done in HEPES buffer (pH 7.3) in the presence (▨) and absence (□) of 50 mM ethanol. Results are expressed as percentages of the basal rate of control (i.e. in the absence of ethanol). The number in parentheses represents the number of experiments. In all the experiments, assays were done in quadruplicate for each treatment indicated.

ods yielded essentially similar results. Synaptosomes from control animals incorporated ^{3}H from *S*-adenosyl-L-([^{3}H]methyl)-methionine at the rate of 67.1 ± 7.0 pmoles/mg protein/hr (mean ± S.E., $N = 8$). The majority of the label in the lipid fraction was found to co-migrate on thin-layer chromatograms with phosphatidylcholine (Fig. 1) and the incorporation was increased by isoprenaline ($IC_{50} \simeq 10^{-5}$ M).

Addition of various concentrations of ethanol to rat brain synaptosomal preparations *in vitro* produced a dose-dependent decrease in the rate of phospholipid methylation. Significant effects being seen with as low a concentration as 10–20 mM. Maximal inhibition was seen with concentrations of 80–100 mM, when the extent of inhibition was 25–30% (Fig. 2).

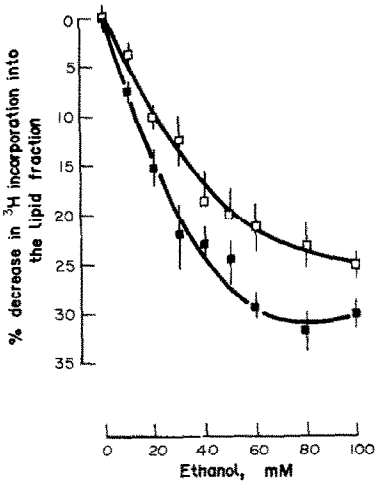

Fig. 2. Effect of various concentrations of ethanol on incorporation of ^{3}H into lipid fraction of rat brain synaptosomes using HEPES (□) and 'artificial CSF' (■) buffers. Results are expressed as % inhibition relative to control. Each point represents the mean ± S.E. of four determinations.

When synaptosomes were obtained from rats which had been exposed to ethanol *in vivo*, alterations in phospholipid methylation were also seen (Fig. 3). When ethanol was administered acutely (2.5 g/kg i.p. 30 min before death) the basal rate of phospholipid methylation was increased by about 15% compared to that of control synaptosomes, but this increase did not reach the $P < 0.05$ level of significance. The administration of ethanol chronically by inhalation for periods of 5–10 days [12] produced rat brain synaptosomal fractions in which the basal rate of phospholipid methylation was significantly increased by about 40% compared to those of control animals. The administration of ethanol by these methods produced blood ethanol concentrations on death (GLC of mixed arterio-venous blood from the neck) in the range 40–80 mM. Addition of 50 mM ethanol to the synaptosomal fractions from chronically treated rats reduced phospholipid methylation to within the control range (Fig. 3). 'Ethanol-withdrawn' rats represent the same group as chronically ethanol-treated animals (10 days), except they were removed from the ethanol vapour chambers for 6–8 hr before death. This time scale was used as it enabled a physical withdrawal syndrome, including marked coarse tremor, postural 'hunching' and piloerection to be observed. Phospholipid methylation in synaptosomal preparations of these animals did not differ from controls either in basal rate or in the inhibition produced on addition of 50 mM ethanol.

Discussion. The findings suggest that the enzymes responsible for the progressive methylation of phosphatidylethanolamine to phosphatidylcholine are sensitive to inhibition by concentrations of ethanol tolerated by mammals *in vivo*. Since these enzymes and their substrates are probably located within the membrane lipid bilayer [3–5] this suggests that this *in vitro* effect of ethanol may be due to some action within the membrane. When synaptosomal

membranes were taken from brains of rats made tolerant to ethanol there was an increased rate of phospholipid methylation, which may reflect either some change in the characteristics of the membrane itself [6–9] or in the enzymes responsible for methylation. The addition of ethanol *in vitro* to these 'tolerant' preparations still produced inhibition of phospholipid methylation suggesting that the basis of tolerance is not a simple exclusion of ethanol from the membrane as has been suggested by partition data [13]. The rapid return of the increased rate of phospholipid methylation in membranes from chronically ethanol-treated animals towards a normal rate on withdrawal of ethanol incicates that, whatever change is responsible, it is relatively short-lived. It seems unlikely that these changes can be ascribed to general alterations in the protein : lipid content of synaptosomal membranes since in previous experiments with this time course of ethanol administration we have observed no alteration in this ratio.

The role of changes in synaptic membrane phospholipid metabolism in ethanol tolerance and dependence has not yet been fully assessed. The increase in phospholipid methylation as assayed *in vitro* on chronic administration of ethanol could be considered as an adaption to the depression of methylation produced by the *in vivo* presence of ethanol. The putative role of phospholipid methylation in β-adrenoceptor : adenylate cyclase coupling [5] and calcium transport [5] would suggest that alterations in these parameters should be associated with ethanol tolerance and some appropriate changes have in fact been described [14, 15]. Recent evidence from other workers [16, 17] also shows increased phospholipid metabolism in brain membranes during the development of ethanol tolerance and dependence but it remains to be seen whether these have important functional effects on synaptic transmission.

These changes may be relevant to those which we have previously described in which reductions in polyunsaturated acyl chains of synaptosomal membrane phospholipids are produced by subacute administration of ethanol to mice [7]. If the methylation of phosphatidylethanolamine to phosphatidylcholine is an important route for incorporation of more unsaturated fatty acids into membrane phospholipids then inhibition of this series of reactions by ethanol could be a factor in our previous findings. Whether such a change is relevant to rapid adaption to ethanol's effects on membranes remains to be seen.

In conclusion we have shown inhibition by ethanol *in vitro* of phospholipid methylation in rat brain synaptosomal fractions. When these preparations are taken from ethanol-tolerant rats phospholipid methylation is enhanced but returns to normal levels 6–8 hr after removal of ethanol.

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