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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1790–1792, 1982.
Printed in Great Britain.

0006-2952/82/091790-03 $03.00/0
Pergamon Press Ltd.

Ethanol and synaptosomal calcium binding

(*Received* 15 *August* 1981; *accepted* 4 *November* 1981)

Alcohols and barbiturates produce diverse alterations in calcium metabolism, and it has been suggested that these effects may mediate some of the pharmacological actions of these drugs [1–3]. Recent studies indicate that ethanol and pentobarbital alter the binding of calcium to biological membranes. *In vitro* addition of relatively low ethanol concentrations (10–100 mM) increases the binding of calcium to a cardiac membrane lipoprotein [4] and to a crude preparation of brain membranes [5]. In a study by Low *et al.* [6], the effects of anesthetic drugs on the binding of calcium to the cytoplasmic surface of erythrocyte membranes were explored. In that study, cells were made permeable to ^{45}Ca by incubation with the calcium ionophore A23187, which allows calcium binding to intracellular sites, and an NaCl–EGTA* wash solution was then used to remove (by displacement and chelation) the ^{45}Ca bound to the external surface. These investigators found that calcium binding to the cytoplasmic surface of erythrocyte membranes is increased by 400 mM ethanol and decreased by higher ethanol concentrations. In contrast to ethanol, pentobarbital (1–7 mM) merely decreases calcium binding [6]. Synaptic mechanisms are important in the actions of alcohols and barbiturates; therefore, our experiments were designed to answer these questions: (1) does *in vitro* addition of ethanol or pentobarbital alter calcium binding to either the cytoplasmic or external surface of brain synaptosomes? and (2) does chronic *in vivo* ethanol consumption alter synaptosomal calcium binding or the effects of *in vitro* addition of ethanol?.

Male BALB/C mice (20–28 g) (Harlan Laboratories, Indianapolis, IN) were decapitated and synaptosomes were isolated from whole brain homogenates by Ficoll gradient centrifugation, as described in previous reports [7, 8]. To study chronic ethanol ingestion, mice were fed a liquid diet conatining 7% (v/v) ethanol or were pair-fed an isocaloric sucrose diet [7]. Binding of ^{45}Ca to the external membrane surface was studied by suspending synaptosomes (0.3 mg protein/ml) in sucrose (300 mM)–Tris (20 mM) buffer (pH 7.6). A 1.7-ml aliquot was placed in a plastic minivial (RPI, Mt. Prospect, IL) that previously had been washed with 1 mM EGTA and rinsed three times with deionized H_2O. After 5 min at 30°, 0.2 ml $^{45}CaCl_2$ solution and 0.1 ml ethanol, pentobarbital sodium (all diluted in H_2O), or H_2O (control) were added. The final concentration of calcium, determined by atomic absorption spectroscopy, was 10^{-5} M. After 10 min at 30°, the vials were supported by 00 rubber stoppers in a Sorvall SS24 rotor and centrifuged at 10,000 rpm for 3 min without refrigeration. The supernatant fraction was immediately decanted, and the vial and pellet surface were rinsed with 1 ml H_2O. Pellets were digested overnight with 0.5 ml of 0.1% SDS; radioactivity was determined by liquid scintillation spectrometry. To estimate the volume of extrasynaptosomal fluid trapped in the vial, some samples in each experiment were incubated with [^{14}C]sorbitol rather than ^{45}Ca [9]. The extrasynaptosomal volume was used to calculate the amount of free ^{45}Ca in each vial, and this value was subtracted from the total ^{45}Ca to give the amount of ^{45}Ca bound.

Calcium binding to intrasynaptosomal sites was determined by the method of Low *et al.* [6]. Synaptosomes were suspended in sucrose–Tris buffer, as described above, and 1.7-ml aliquots were incubated for 20 min at 30° with 10 μM A23187 (Calbiochem-Behring Corp., La Jolla, CA) (dissolved in 2 μl DMSO), 0.1 ml ethanol, pentobarbital, or H_2O (control) and 0.2 ml ^{45}Ca–EGTA solution. The free concentration of calcium was calculated as 7 μM [10]. This value, however, did not take into account the binding of calcium to A23187 or to the membranes. Although A23187 may release calcium from synaptosomal storage sites (mitochondrial and non-mitochondrial), changes in free calcium concentration would be minimized by the EGTA buffer. The suspension was then diluted with 8 ml of 140 mM NaCl, 20 mM Tris, and 5 mM EGTA (pH 7.5) and rapidly filtered through Whatman GF/C discs which were washed with another 8-ml aliquot. Liquid scintillation spectrometry was used to determine radioactivity on the discs, the phenol method [11] to determine protein, and Student's *t*-test for paired observations to statistically evaluate drug effects.

In vitro addition of ethanol enhanced calcium binding to intrasynaptosomal sites (Fig. 1). Ethanol concentrations of 12–50 mM increased binding by about 30%; higher con-

* Abbreviations: EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate; SDS, sodium dodecylsulfate; and DMSO, dimethylsulfoxide.

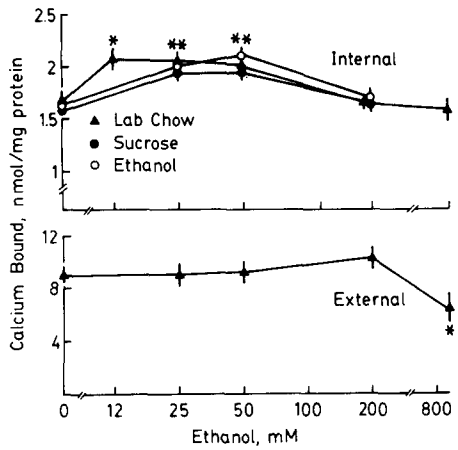

Fig. 1. Effects of *in vitro* and *in vivo* ethanol treatments on the binding of ^{45}Ca to brain synaptosomes. Upper panel: intrasynaptosomal calcium binding. Synaptosomes were isolated from mice given lab chow (▲), and ethanol liquid diet (○), or an isocaloric sucrose liquid diet (●). Ethanol was added *in vitro* at the concentrations given on the abscissa. Vertical bars represent ± S.E.M., N = 11–36. Lower panel: Binding of ^{45}Ca to the external surface of synaptosomes isolated from mice given lab chow. Ethanol was added *in vitro* at the concentrations given on the abscissa. Vertical bars represent ± S.E.M., N = 4–12. In both panels a single asterisk (*) signifies a significant effect of ethanol, $P < 0.05$, and a double asterisk (**), $P < 0.01$.

centrations did not alter binding. The same intrasynaptosomal binding of calcium and effect of ethanol were seen, whether synaptosomes were from mice given free access to lab chow or an ethanol-containing liquid diet or from those pair-fed an isocaloric sucrose diet. Consumption of ethanol also failed to change the binding of calcium (0.005 to 1 mM) to the external synaptosomal surface (data not shown). In contrast to the effects on intrasynaptosomal binding, *in vitro* addition of low concentrations of ethanol did not affect binding of calcium to the external surface of the synaptosomes; a high concentration, however, reduced binding (Fig. 1). Similar ethanol effects on external calcium binding were observed with calcium concentrations of 0.001, 0.01, 0.1, and 1 mM (data not shown).

Pentobarbital had no effect on intrasynaptosomal calcium binding, but at the highest concentration tested (0.5 mM) it produced a small, but very consistent, decrease in calcium binding to the external synaptosomal surface (Table 1).

These results demonstrated that low, physiologically relevant concentrations of ethanol selectively enhanced intrasynaptosomal calcium binding without affecting binding to the external surface. Low *et al.* [6] have reported that ethanol enhances calcium binding to intracellular sites of erythrocytes but, compared with our study, the enhancement of erythrocyte binding was less and required 10- to 30-fold higher concentrations of ethanol. In our study, the increase in intrasynaptosomal calcium binding was similar for ethanol concentrations of 12–50 mM; this resembles the effects of ethanol on crude preparations of brain membranes, where 10–100 mM ethanol produces a consistent 20% increase in calcium binding [5]. Likewise, ethanol has been shown to produce biphasic effects on synaptosomal calcium transport and membrane fluidity, with a range of low concentrations producing similar effects [7, 12, 13]. In the latter studies, however, high concentrations of ethanol (e.g. 800 mM) produced marked effects, whereas in the present study a high concentration did not affect intrasynaptosomal calcium binding.

Table 1. Effects of pentobarbital on the binding of calcium to synaptosomes*

Addition	Internal binding	External binding
H_2O	1.6 ± 0.1	11.3 ± 0.5
Pentobarbital		
0.12 mM	1.7 ± 0.2	ND†
0.25 mM	ND†	10.5 ± 0.1
0.50 mM	1.8 ± 0.1	9.9 ± 0.6‡

* Values represent nmoles calcium/mg protein, mean ± S.E.M., N = 4–6.
† Not determined.
‡ Significantly different from H_2O, $P < 0.01$.

The present results do not allow a molecular description of the calcium binding sites affected by ethanol; however, we have some information about these sites. First, in the absence of the ionophore, there was some calcium binding, which suggests that the chelation–displacement step did not completely remove external ^{45}Ca. This residual binding, however, was not increased by ethanol (data not shown). These results, together with those in Fig. 1, indicate that ethanol enhanced intra-, not extra-, synaptosomal binding. Second, this binding did not represent intrasynaptosomal sequestration of calcium, because these uptake processes require ATP [12] and there was no energy source in our incubation mixture. Also, *N*-ethylmaleimide and oligomycin, inhibitors of non-mitochondrial and mitochondrial calcium sequestration, respectively [12], did not alter intrasynaptosomal calcium binding (data not shown). These conditions also eliminate any involvement of membrane potentials in the ethanol effects. Third, the measured binding cannot be attributed to free ^{45}Ca trapped in the synaptosomes. We determined the intrasynaptosomal water volume [9] and calculated the amount of ^{45}Ca in that volume; this was negligible compared to the total amount of ^{45}Ca bound. Thus, ethanol may enhance calcium binding to the cytoplasmic surface of the plasma membrane, to the surface of intrasynaptosomal organelles, or to soluble proteins.

Consumption of an ethanol-containing liquid diet for 16 days has been reported to cause a marked reduction in the binding of calcium to a crude preparation of rat brain membranes and a reduction in the *in vitro* effects of ethanol on this binding [5]. Chronic consumption of ethanol has also been noted to reduce the binding of calcium to synaptic plasma membranes [14]. In contrast, we found that ethanol consumption had no effect on synaptosomal calcium binding. These apparently conflicting findings may have been due to the difference in (1) species, (2) membrane preparations, and (3) the length of time alcohol was ingested.

The effects of pentobarbital on intrasynaptosomal calcium binding were clearly different from those of ethanol, as pentobarbital failed to enhance binding. A similar distinction between the two drugs has been noted with regard to erythrocyte calcium binding [6]. Distinctions between barbiturates and ethanol also have been noted in genetic [15], neurochemical [16, 17], and neurophysiological [17] studies.

In summary, we found that *in vitro* addition of concentrations of ethanol equivalent to those producing intoxication *in vivo* enhanced intrasynaptosomal binding of calcium, whereas higher concentrations (equivalent to anesthetic or lethal levels) had no effect. This suggests that enhancement of calcium binding may be one of the factors responsible for the disinhibition associated with low doses of ethanol. The increased intrasynaptosomal calcium binding could modulate synaptic activity by means of two mechanisms: (1) a reduction in intrasynaptosomal levels of free calcium could alter the activity of calcium-dependent

enzymes and perhaps reduce neurotransmitter release, and (2) increased binding of calcium to the cytoplasmic surface of the synaptic plasma membrane could change the physical properties (fluidity, lateral phase separations) of the inner half of the membrane bilayer, and these membrane property changes could alter membrane-bound enzymes and membrane transport [18–22]. Thus, in addition to its direct effects on membrane physical properties [13, 23], ethanol may indirectly alter synaptic membrane properties by changing calcium binding.

Acknowledgements—This work was supported by the Medical Research Service of the Veterans Administration and by Public Health Service Grant DA-02855. We thank Velma Henthorne for typing the manuscript.

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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1792–1795, 1982.
Printed in Great Britain.

0006-2952/82/091792-04 $03.00/0

Microsomal enzyme deficiencies in the Gunn rat

(*Received* 20 *June* 1981; *accepted* 26 *October* 1981)

Gunn rats, in their jaundiced homozygous phenotype (jj or −/−), lack liver UDP glucuronyl transferase (EC 2.4.1.17) activity for bilirubin. Heterozygous animals (Jj or +/−) have demonstrable transferase activity for bilirubin which, however, is significantly less than the activity present in outbred normal rats (JJ, +/+) [1–3]. *In vitro* glucuronidation activities for other aglycones in the jj animal vary from markedly reduced to normal levels [1–7]. The deficiency in the Gunn rat is an animal model of the human disorder known as the Crigler–Najjar syndrome, in which bilirubin glucuronyl transferase (BGT) activity is also lacking and is transmitted as an autosomal recessive trait [8]. This transferase deficiency in both rat and man has been presumed to reflect a single gene defect in the synthesis of this enzyme protein. An alternative hypothesis that might explain these enzymatic abnormalities is a genetically controlled alteration of the membrane in which glucuronyl transferase is compartmented. Such an abnormality could either alter enzyme conformation or decrease substrate penetration through the microsomal membrane, thereby decreasing enzyme activity. If a membrane defect is the primary abnormality, other enzymes also located in microsomes might exhibit deficiencies which would segregate like that of the glucuronyl transferase in the Gunn rat. With this in mind, the activity of aminopyrine demethylase, an hepatic microsomal cytochrome P-450, mixed-function oxidase (EC 1.14.14.1), was assayed in male jj, Jj and JJ Gunn rats.

Materials and methods

The aminopyrine demethylase assay was modified from Matsubara *et al.* [9]. Incubation mixtures contained 10 mM aminopyrine, 0.4 mM NADP, 5 mM glucose-6-phosphate,