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EFFECTS OF ETHANOL ON RAT BRAIN (Na + K)ATPase FROM NATIVE AND DELIPIDIZED SYNAPTIC MEMBRANES

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Abstract—The role of lipids in the effect of ethanol on synaptosomal (Na + K)ATPase was studied using native and partially delipidized synaptosomal membranes from control and alcoholic rats. A biphasic effect of alcohol was observed with the (N + K)ATPase from control membranes. Ethanol at low concentrations (<100 mM) appears to enhance the enzyme activity, but at higher concentrations (>300 mM) was inhibitory. The biphasic response to ethanol was also observed with the (Na + K)ATPase isolated from alcoholic animals; however, in this case the enzyme showed a resistance to the inhibitory effect of ethanol. Delipidization of synaptic membranes with Lubrol WX or phospholipase A practically abolishes the effects of alcohol on (Na + K)ATPase from both control and alcoholic animals. It thus seems that the effects of ethanol are due mainly to their interaction with the lipids surrounding the enzyme. Furthermore, addition of ethanol to native membranes did not change the V_{max} and K_m for K^+. However, when ethanol at the same concentration was added to delipidized membranes, a decrease in K_m with no change in V_{max} was observed. Ethanol under these conditions apparently interacts also with the enzyme protein. On the other hand, chronic ethanol intake produces an increase of both V_{max} and K_m for K^+. However, when alcohol was added *in vitro*, there were no changes in the kinetic parameters of either native or delipidized membranes. These data indicate that although the effects of ethanol on synaptosomal (Na + K)ATPase are mainly due to its interaction with the lipid microenvironment of the enzyme, a direct ethanol action on the enzyme protein also occurs. Our data further suggest that chronic ethanol treatment alters enzyme sensitivity to the effect of ethanol which may be related to the membrane–lipid composition and/or to changes in the conformation of the enzyme protein.

Ethanol has been shown to affect a large number of interrelated membrane systems, especially in the central nervous system [1]. At high doses it produces a fluidification and a disorganization of the membrane structure [2, 3]. Chronic ethanol consumption appears to produce compensatory changes in the membrane constituents, mainly in the lipid fraction [4, 5], which serve to increase the rigidity of the membrane [6]. Since the lipid composition of the membrane has a direct influence on membrane-bound enzymes, changes in membrane lipids could alter the behavior of some of these enzymes. We have shown that prolonged ethanol intake increases the activity of some synaptic-bound enzymes, alters lipid–protein interactions and increases the cholesterol content of these membranes [7, 8]. However, the question whether the changes in enzymatic activity are due to a direct effect of ethanol on the proteins or to an alteration in membrane lipids is not settled.

It is widely accepted that lipid performs an essential role in the activity of the plasma membrane (Na + K)ATPase. Ethanol at high concentrations inhibits its activity [9], but chronic ethanol intake induces a resistance to this inhibitory effect of ethanol [10]. Relative to this, some authors have suggested that the inhibitory effect of ethanol occurs secondarily to changes in the properties of the lipids surrounding the enzyme [10, 11], while others postulate that ethanol interacts with the enzyme protein preventing the formation of the K^+-sensitive enzyme by cation binding [12, 13]. In a recent report it has been demonstrated that the inhibition of (Na + K)ATPase by alcohol depends to a considerable extent on its ability to partition into cell membranes, although it does not appear to account entirely for the enzyme inhibition, suggesting a possible direct action of ethanol on the enzyme protein [14].

The aim of the present work has therefore been to investigate whether the effect of ethanol on the membrane-bound (Na + K)ATPase is due to a direct interaction with the enzyme protein or whether it is due to ethanol-induced changes in the properties of the lipids surrounding the enzyme, or both. We have used native and delipidized synaptic membranes, to (1) determine the enzyme activity after addition of different concentrations of ethanol, (2) investigate some kinetic parameters of the (Na + K)ATPase in order to further elucidate the direct action of ethanol on the mechanism of this enzyme, (3) draw Arrhenius Plots of the (Na + K)ATPase to assess possible changes in lipid–protein interaction. These points have been studied in synaptic membranes from both control and chronic alcoholic animals in order to elucidate at the same time, the role of lipids in the alteration of the enzyme sensitivity to ethanol which occurs after alcohol-treatment [10].

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MATERIALS AND METHODS

Animal treatment. Male Wistar rats with an average initial body weight of 150 g were fed for 8 weeks a liquid diet [15] in which ethanol provided 36% of total calories, 16% protein and 35% fat. Pair-fed control rats were given a similar diet for the same period, except that carbohydrates replaced ethanol isocalorically.

During the experimental period, rats were housed in plastic boxes, 3 animals per box, at 22°, 60% humidity, on a 12 hr light/dark cycle.

Preparation of synaptic membranes. Rats were sacrificed by decapitation and brains were quickly removed. The cerebellum and brainstem were discarded and the cortex was homogenized (10% w/v) in cold 0.32 M sucrose, pH 7. Synaptosomes were prepared according to Cotman [16] as described previously [7]. Synaptic membranes were purified following the procedure of Ross *et al.* [17], using two to three brains in each preparation. Purified membranes were suspended in 20 mM Tris–HCl (0.5 mM dithioerythritol) pH 8.5, frozen overnight (−30°), thawed and used for enzyme assay and other procedures.

Enzymatic measurements and other procedures. (Na + K)ATPase was measured as previously described [7]. Two reaction mixtures were assayed simultaneously. One reaction mixture contained in 1 ml: 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl, 5 mM Tris–ATP and 0.1 ml of a thawed suspension of synaptosomes (25–60 μg). The second mixture was essentially the same except that NaCl and KCl were omitted and 1 mM ouabain was added. Reaction mixtures were preincubated for 5 min at 37° and the reaction was initiated by the addition of Tris–ATP. After further incubation for 15 min, the reaction was terminated by the addition of ice-cold 10% TCA (1 ml). Inorganic phosphate was determined in the supernatant fraction by the method of Gomori [18]. (Na + K)ATPase activity was calculated as the difference between the quantity of inorganic phosphate liberated in the presence of Na^+ and K^+, and in the absence of Na^+ and K^+ and the presence of ouabain, in the incubation medium.

To assess the K_m for potassium of (Na + K)ATPase from synaptic membranes, the concentration of potassium (KCl) in the incubation mixtures was varied between 0.5 and 20 mM. To assess Arrhenius Plots of the (Na + K)ATPase, a thawed suspension of synaptic membranes (50–60 μg/ml of protein) was incubated with the reaction mixture for 20 min (10–21°) or 15 min (25–40°).

Experiments were also performed to determine the effect of ethanol, added *in vitro*, on synaptosomal (Na + K)ATPase from ethanol-treated and control rats. In these experiments, ethanol (25–200 mM) was added to the incubation mixtures before the addition of Tris–ATP.

Protein was determined by the method of Lowry *et al.* [19].

Lines were fitted to the data points in Arrhenius Plots and in the Lineweaver–Burk plots by regression analysis. Statistical comparisons were made by the Student *t*-test.

Removal of phospholipids. (1) Phospholipase A treatment was according to the procedure of Fleischer [20]. Synaptosomal protein (2.5 mg) was suspended in 0.5 ml of medium containing 156 μg phospholipase A, 10 mM glycylglycine buffer pH 7.4, 2 mM $CaCl_2$, 0.5% serum albumin in 10 mM glycylglycine pH 7.4 and 50 mM sucrose. The reaction mixture was incubated 2 min at 37°, the reaction was begun by the addition of the phospholipase A and incubation was for 20 min in a shaking water bath. The reaction was terminated by dilution of the samples with 9 vol. of cold 1.0% serum albumin in 0.05 M glycylglycine pH 7.4. The mixture was centrifuged at 45,800 *g* for 30 min. This preparation was referred to as "once washed". The residue was resuspended in 10 vol. of the same wash solution using a Potter–Elvehjem homogenizer. This procedure was repeated twice before a final wash with 0.25 M sucrose. Finally, the pellet was suspended in 20 mM Tris–HCl, 0.5 mM DTT, pH 8.5, at a protein concentration of 1–2 mg/ml.

(2) Lubrol-WX-treated membranes: membranes were partially solubilized according to the method outlined by Robinson [21]. Synaptosomal protein (0.5 mg) was suspended at room temperature in 0.55 ml of medium containing 0.05% (w/v) Lubrol-WX, 0.07 M KCl, 0.01 M Tris–HCl (pH 7) and 40% (v/v) dimethyl sulfoxide. The incubation, which proceeded for 30 min, was terminated by adding 0.1 ml of 0.03 M $MgCl_2$, 0.1 ml 1% (w/v) lecithin suspension and 1 ml of 1.2 M sucrose, all solutions at 0–5°. This mixture was diluted with 25 ml of 0.25 M sucrose and centrifuged at 30,000 *g* for 30 min. The resultant pellet was washed once, then resuspended in 20 mM Tris–HCl (DTT 0.5 mM), pH 8.5, at ~1 mg/ml.

RESULTS

Effect of Lubrol WX and phospholipase A treatments on synaptic membrane (Na + K)*ATPase*

The activity of the (Na + K)ATPase is dependent upon the amount and composition of the membrane lipids. Treatment with phospholipases or lipid extraction with detergents frequently lead to either partial or total inactivation. Thus, under our conditions, after the treatment with phospholipase A or Lubrol WX, the activities remaining were ≈20% and 16%, respectively, of that of native control membranes, i.e. 27.1 ± 0.05 μmol Pi/hr/mg prot.

On the other hand, when these treatments were carried out with membranes isolated from alcoholic animals and the same conditions, the remaining activity was 35% with phospholipase A and 17% with Lubrol WX treatment. The specific activity of the (Na + K)ATPase from native alcohol treated membranes was 33.5 ± 0.08 μmol Pi/hr/mg prot.

Effect of ethanol on (Na + K)*ATPase from native and delipidized synaptic membranes.*

As shown in Fig. 1a, control synaptosomal (Na + K)ATPase exhibited a biphasic response to ethanol. At low concentrations (<100 mM), ethanol enhanced, but the activity was inhibited at concentrations higher than 200 mM. This biphasic effect of ethanol was eliminated when the membranes were

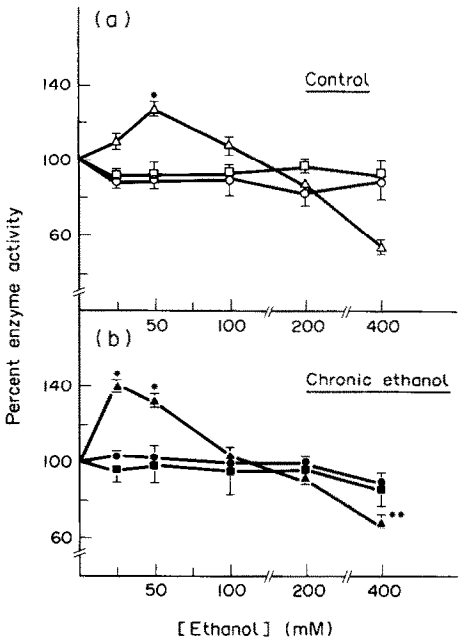

Fig. 1. Effect of ethanol on synaptosomal (Na + K)ATPase from control (a) and chronic ethanol-treated animals (b). Native synaptic membranes (25 μg prot/ml) (△, ▲) or membranes treated with Lubrol WX (40 μg prot/ml) (○, ●) or phospholipase A (40 μg prot/ml) (□, ■) were added to the incubation assay containing 0–400 mM ethanol (see Materials and Methods). Each point represents the average of 4 different experiments done in duplicate ± SD. * $P \leq 0.01$ vs the activity without alcohol. ** $P \leq 0.01$ vs the corresponding control.

treated with either Lubrol or phospholipase A. On the other hand, when synaptic membranes were isolated from chronic alcoholic animals, the (Na + K)ATPase activity also showed a biphasic response to ethanol Fig. 1b. However, in this case the peak of activation was at 25 mM ethanol; at higher concentrations (>300 mM; Fig. 1 and results not presented) the enzyme was more resistant to the inhibitory effect of ethanol. When the membrane were delipidized, the effect of ethanol was abolished (Fig. 1b) as in control membranes.

Effect of lipid removal on temperature dependence of (Na + K)-ATPase activity

Figure 2 (left panel) shows the variation, with changes in incubation temperature, of the synaptosomal (Na + K)ATPase activity from control animals. A biphasic Arrhenius plot is observed with a discontinuity of the plot at 28°. However, when the membrane lipids were removed by phospholipase A or Lubrol WX treatments, the transition temperature (TT) shifted to 21° in both cases. Both treatments alter the activation energies (E_a), but in a different way. Thus, Lubrol produced a decrease in E_a both below and above TT, while phospholipase digestion induced a significant change in E_a only above the TT. This difference in effect on E_a may be due to specific differences in lipid removal by the two treatments. Figure 2 (right panel) also shows, in agreement with previous results, that chronic ethanol consumption produced an increase in the transition temperature of the (Na + K)ATPase to 33.7° and possibly in E_a both below and above TT. As in controls, delipidization of the membranes lowered the TT, but the difference in TT between alcohol and control animals was less than in native membranes. Furthermore, after lipid removal, the E_a for both treatments changed with respect to the values obtained with control membranes.

Effect of ethanol on Lineweaver–Burk plot of (Na + K)ATPase from native and delipidized synaptic membranes

The double-reciprocal plot of (Na + K)ATPase from native membranes vs potassium concentration, shows that the addition of 0.1 M ethanol (a non-inhibitory concentration with respect to the activity without ethanol, see Fig. 1) did not change substantially either the K_m or the V_{max} (Fig. 3, left panel, N). On the other hand, membrane treatment with Lubrol WX (Fig. 3, left panel, L) and phospholipase A (Fig. 3, left panel, Ph) lowered the V_{max} from 26.6 to 3.45 and 5.26 μmol Pi/hr/mg prot., respectively, but only Lubrol appeared to change somewhat the K_m value (from 1.43 to 1.01 mM). However, when delipidized control membranes were incubated with 0.1 M ethanol, there were no apparent changes in K_m, but V_{max} decreased even more, at least after Lubrol treatment (from 3.45 to 2.5 μmol Pi/hr/mg prot.). Thus, ethanol in the delipidized control membranes may act as a non-competitive inhibitor.

Figure 3 (right panel) shows the Lineweaver–Burk plots of the (Na + K)ATPase reaction velocities against K^+ concentration, from chronic alcoholic synaptic membranes. As shown with prolonged alcohol consumption the K_m and V_{max} values for K^+ were higher than with control membranes (K_m, 1.85 mM; V_{max}, 33.3 μmol Pi/hr/mg prot.). Delipidation of these membranes caused a marked decrease in V_{max} (4.54 for Lubrol and 11.76 μmol Pi/hr/mg prot. for phospholipase) and possibly changed marginally the K_m value when phospholipase was used. Furthermore, when 0.1 M ethanol was added to the delipidized membranes from alcohol-treated rats, the effect was different than with control delipidized membranes, since in this case there were only minor changes in V_{max}.

DISCUSSION

It has been postulated that ethanol alters the packing of molecules in the phospholipid bilayer and increases fluidity of the cell membrane [2]. Functional consequences of this disruption are probably mediated by proteins embedded in the fluid lipid environment. However, the question of whether lipids or proteins are the primary target of ethanol action has not been settled.

(Na + K)ATPase is an important membrane-bound enzyme, whose activity is inhibited by ethanol *in vitro*. It has been assumed that the inhibitory effect of ethanol on (Na + K)ATPase activity occurs secondarily to ethanol-induced changes in the lipid microenvironment of the enzymes [10, 11]. Our results indicate that while ethanol at high concentrations (>300 mM) inhibited the (Na + K)ATPase, at lower concentrations the

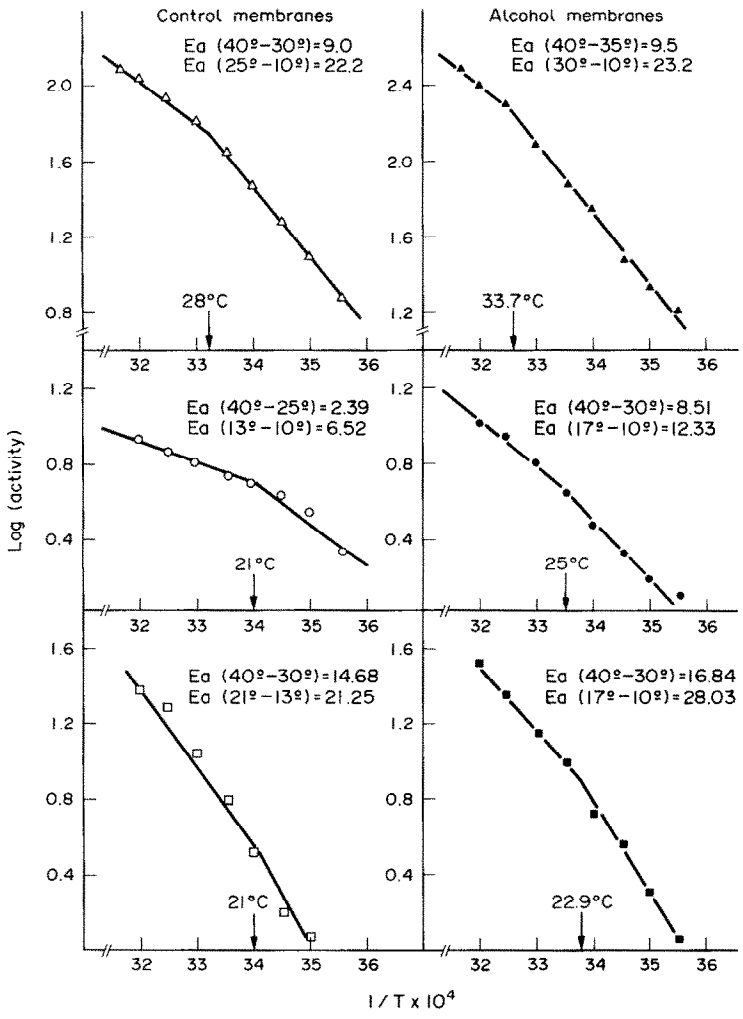

Fig. 2. Effect of Lubrol WX and phospholipase A treatment on the Arrhenius plots of (Na + K)ATPase from control (left panel) and ethanol-treated (right panel) synaptic membranes. Enzyme protein (50–60 μg ml) from native and treated synaptic membranes was assayed at different temperatures (see conditions in Materials and Methods). The activity values are obtained from initial rate of reaction and are expressed in μmoles Pi/hr/mg prot. Symbols as in Fig. 1. Each point represents the average of 8 different experiments (from 4 different preparations) ± SD. Lines were fitted to the data points in Arrhenius Plots by regression analysis.

enzyme activity was enhanced. These results are in agreement with Sun *et al.* [22, 23], although in an earlier study these authors did not find the biphasic effect [9]. They suggest that the discrepancy may be due to the use of an enzyme preparation (synaptosomes instead of synaptic membranes) [22].

The present data also demonstrate that chronic ethanol consumption decreased the sensitivity of (Na + K)ATPase to ethanol inhibition. This type of resistance to the effect of ethanol on membrane-related functions has been considered to be a cellular or molecular analog of tolerance [10, 24] and may be related to changes in the physico-chemical composition of the membrane lipids. In fact, when the membranes were delipidized, the biphasic effect of alcohol as well as the difference in sensitivity of (Na + K)ATPase to alcohol inhibition observed in alcoholic vs control animals, practically disappeared.

In contrast with our data, Lin has reported that Lubrol WX increase (Na + K)ATPase sensitivity to ethanol. The discrepancies may be due to the concentrations of detergent (lower than 0.07%) and the conditions of Lubrol treatment (Lubrol was added to the assay mixtures) [25].

Another way to clarify the effect of alcohol on the lipids surrounding the enzyme protein is by the study of Arrhenius plots. In this respect, recent work in our and other laboratories [7, 10] has indicated that ethanol alters the lipid–protein interactions of (Na + K)ATPase as measured by alterations in the transition temperature of Arrhenius plots of this enzyme. Decreases in transition temperature (TT) are induced by ethanol added *in vitro* [7, 10, 26] and chronic ethanol treatment produced an increase in the break temperature [7], which confirms the data of the present work and which may be a response to

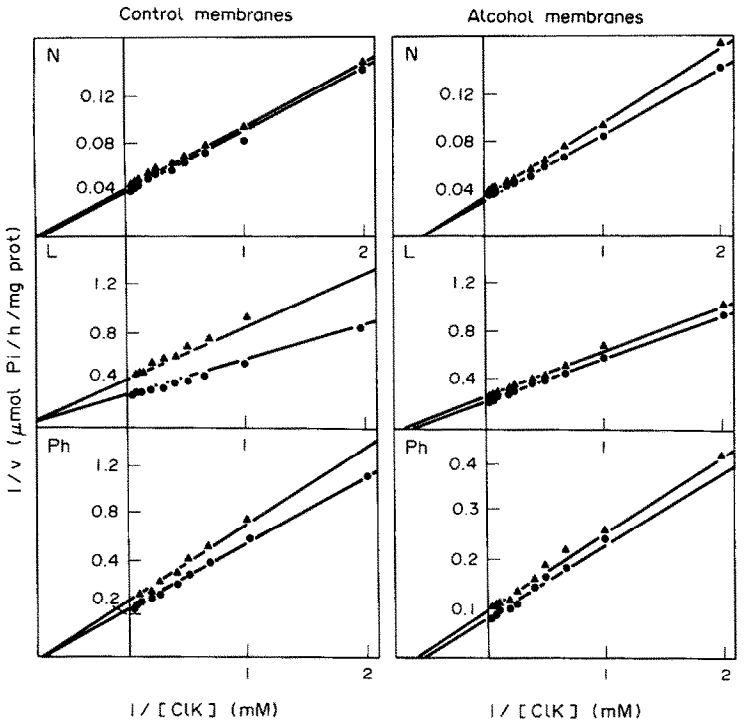

Fig. 3. Effect of ethanol on double reciprocal plots of synaptosomal (Na + K)ATPase activity against K^+ concentrations from control (left panel) and chronic alcoholic animals (right panel). N = native membranes; L, Ph = membranes treated with Lubrol WX or phospholipase A. Enzyme protein from native (25 μg/ml) and treated (40 μg/ml) membranes was assayed in the absence (●) or in the presence of 0.1 M ethanol (▲) at different concentrations of K^+. Each value is an average of five experiments done in duplicate. Lines were fitted to the data points in Lineweaver–Burk plots by regression analysis.

changes in membrane lipid composition [8, 27, 28]. However, Levental and Tabakoff [10] and Rangaraj and Kalant [26] have reported a lower TT for the (Na + K)ATPase from animals fed an ethanol diet for 7 or 20 days, respectively. As we discussed in the previous study [7], the discrepancies may be due to the duration of the treatment. Thus, we found a lower or higher TT depending on whether the animals were maintained on alcohol for 2 or 4–8 weeks, respectively. On the other hand, extended treatment with the control liquid diet could explain the higher TT (28°) which we obtained for the control group.

Since changes in the TT of Arrhenius plots specifically reflect the boundary lipids immediately adjacent to the enzyme [29], lipid extraction with detergents or phospholipases affects the TT and in some cases abolishes the discontinuities [30, 31]. Under our conditions of membrane treatment with Lubrol WX and phospholipase A, the TT of (Na + K)ATPase for both control and alcohol fed animals decreased, although the temperature dependence remained greater in the ethanol treated membrane. In this respect, since phospholipase A digestion seems to cause mainly a hydrolysis of certain phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylinositol) [31], which were increased after chronic ethanol consumption [28], it is possible that this delipidization treatment could affect synaptic membranes from control and alcoholic animals differently.

Although our data indicate that ethanol effects on (Na + K)ATPase are mainly mediated by an interaction with the lipids surrounding the enzyme, we also demonstrate that ethanol may interact with the enzyme protein. This is supported by the fact that when 0.1 M ethanol was added *in vitro* to native membranes, no change was observed in the V_{max} or K_m for K^+ of (Na + K)ATPase from control native membranes, but when the same alcohol concentration was added to delipidized membranes, a decrease in V_{max} with no change in K_m was noted. Ethanol under these conditions seems to interact with the protein, acting as a non-competitive inhibitor. Although such studies on delipidized membranes have not been published, in a kinetic study of the (Na + K)ATPase from native membranes it has been shown that ethanol reduced the affinity or increased the K_m for K^+. The discrepancies of these studies with our results, from native membranes, may be due to the ethanol concentration used (higher than 0.1 M) [10, 33] and the enzyme preparation [34, 35]. Our findings are in agreement with Nhamburo *et al.* [14] who reported that ethanol can inhibit (Na + K)ATPase activity by partitioning into the membrane lipids surrounding the enzyme, but they suggest that a direct interaction of ethanol with the protein might also be involved in the inhibition.

It is known that alterations in the lipid environment of a membrane-bound enzyme may be associated with changes in such kinetic parameters as V_{max}

and K_m for substrate [36]. And indeed, delipidation or addition of solvent to disrupt lipid–protein interactions is accompanied by a decrease of both V_{max} and K_m [37], as we have confirmed here. Chronic ethanol consumption in addition to altering the membrane lipids, also induced an increase in both V_{max} and K_m relative to control values. However, when ethanol was added *in vitro* to native and delipidized membranes, we found no significant changes in the kinetic parameters. A possible interpretation for this might be that although the delipidization treatment was the same as in controls, "alcoholic" membranes might still retain some lipids which prevent the alcohol from interacting with the enzyme protein. Another possibility is that the conformation of (Na + K)ATPase might be changed so that it would be more difficult for ethanol to interact.

In summary, these findings support the view that, although the effects of ethanol on (Na + K)ATPase are mediated mainly by an interaction with the lipids surrounding the enzyme, there also seems to be a direct ethanol action on the enzyme protein. Our findings suggest further that chronic ethanol treatment alters enzyme sensitivity to the effect of ethanol, which may be due to an alteration of the membrane–lipid composition and/or to a change in the conformation of the enzyme protein.

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