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INVOLVEMENT OF THE LIPID AND PROTEIN COMPONENTS OF $(Na^+ + K^+)$-ADENOSINE TRIPHOSPHATASE IN THE INHIBITORY ACTION OF ALCOHOL

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Abstract—The relative involvement of the lipid and protein moieties of $(Na^+ + K^+)$-activated adenosine triphosphatase in the inhibitory action of alcohol on this enzyme was investigated, using an enzyme preparation derived from the cerebral cortex of mice. Two classes of lipids are envisioned to be associated with this enzyme. One is essential for the activity of the enzyme, while the other is not. Purification or treatment with the nonionic detergent Lubrol WX increased the ATPase sensitivity to ethanol, possibly as a result of the removal of hindering lipids and contaminating proteins from the enzyme. Delipidization with deoxycholate removed both the essential and bulk lipids, and drastically reduced the activity of the enzyme. This could be subsequently reactivated with phosphatidyl serine (PS). The ethanol sensitivity of the reactivated ATPase varied with the ratio of PS to protein, and is most sensitive at 12.5μmoles PS P_i/mg when it is half-reactivated. Increasing the PS concentration cannot completely reverse the inhibition of ethanol on the reactivation. These data suggest that ethanol inhibits $(Na^+ + K^+)$-ATPase activity by interacting with its protein moiety. Furthermore, the sensitivity of the enzyme to ethanol is probably related to its conformation, which, in turn, is affected by its essential lipid.

General anesthetics and cell membranes have long been thought to interact because they are both hydrophobic [1]. One such anesthetic, alcohol, has been shown to increase the fluidity of membrane lipids [2, 3] and the leakage of Na^+ and K^+ across lipid bilayers [4]. It is believed that these changes occur because the state and property of the lipids have been altered [2, 3, 5]. Since integral membrane proteins are also hydrophobic, the possibility that they are involved in the action of alcohol should be investigated.

Previous studies have shown that alcohol might inhibit $(Na^+ + K^+)$-activated adenosine triphosphatase [$(Na^+ + K^+)$-ATPase] activity by altering its conformation [6, 7]. The enzyme is membrane-bound and requires phospholipids for activity [8, 9]. These lipids can be removed, leaving the relatively inactive protein moiety. A study of the effect of alcohol on this enzyme associated with different amounts of lipids could shed some light on the mechanism of alcohol action.

Detergents can stimulate $(Na^+ + K^+)$-ATPase activity and produce a peak activation near their critical micelle concentration (cmc) [10, 11]. They are thought to remove hindering lipids from the ATPase and expose more enzyme sites [10, 11]. It is possible that the enzyme will become more sensitive to the alcohol effect following treatment with a detergent. This paper shows that relatively low concentrations of alcohol can suppress the activation of $(Na^+ + K^+)$-ATPase by the nonionic detergent Lubrol WX, and that the enzyme is most sensitive to alcohol inhibition when its delipidized preparation is half-reactivated with phosphatidyl serine (PS).

MATERIALS AND METHODS

Ten-week-old male mice of the DBA/2J strain were purchased from Jackson Laboratory, Bar Harbor, ME. They were housed four per cage and acclimated to laboratory conditions (8-hr dark period per day at 21°) for at least 1 week before use. They had access to Purina Lab Chow and water *ad lib.* Analytical grade sodium chloride, potassium chloride and magnesium chloride were purchased from the Baker Chemical Co., Phillipsburg, PA. Ox-brain phosphatidyl serine (PS) and egg-yolk phosphatidyl choline (PC) were products of Serdary Laboratories, Ontario, Canada. All other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO, and were of the highest grade available.

Enzyme fractions were prepared at 0–4° unless stated otherwise. The mice were killed by cervical dislocation and the cerebral cortex was rapidly removed. It was immediately homogenized 1:20 (w/v) in Tris–Cl (10 mM, pH 7.4) containing 0.25 M sucrose in a Pyrex glass homogenizer. Ten additional reciprocal strokes were performed after the tissue mass had been dispersed. The homogenate was centrifuged at 120 *g* for 10 min, and the supernatant fluid was filtered through four layers of cheesecloth. This level of enzyme preparation is referred to as the low-speed fraction, and was always used within 1 hr of preparation. Microsomal, NaI-treated and delipidized enzymes were prepared by published procedures [8, 12–14] and stored at −70° for 2 weeks without loss of enzyme activity.

The activities of total ATPase, Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase were assayed as described pre-

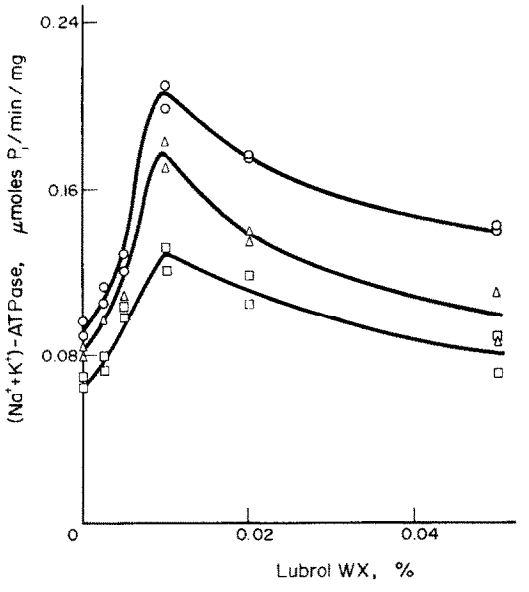

Fig. 1. Ethanol suppression of the activation of $(Na^+ + K^+)$-ATPase by Lubrol WX. $(Na^+ + K^+)$-ATPase activity was obtained by subtracting Mg^{2+}-ATPase activity from the simultaneously assayed total ATPase activity [6]. Both ATPase assay mixtures without (○) or with 0.05 M (△) and 0.5 M (□) ethanol were mixed with the low-speed enzyme preparation (39 μg protein/assay tube) and Lubrol WX to various concentrations. The assay mixtures were equilibrated at 37° for 5 min, and the reactions were initiated by the addition of Tris–ATP. Each assay was duplicated, and the results were reproducible using different enzyme preparations.

viously [6]. One unit of ATPase activity is that amount of enzyme which produces 1 μmole of inorganic phosphate (P_i) from ATP per min at the present assay conditions. When Lubrol WX, alcohol or phospholipid was present, it was added to the assay mixture before the initiation of the enzyme activity with Tris–ATP. PS and PC vesicles were prepared by sonication with distilled water as described [12]. They were adjusted to a concentration of 15 mM Pi and stored under nitrogen at 4°.

Lipids in the enzyme preparations were extracted three times with 20 vol. of chloroform–methanol (2:1, v/v) at room temperature. Each extraction was 20 min. The combined extracts from each enzyme preparation were evaporated to dryness using a Rotavapor (Buchi/Brinkman) and the amounts of phosphorus (Pi) were measured [15]. Protein was determined by the procedure of Lowry *et al.* [16] using bovine serum albumin as the standard.

RESULTS

Figure 1 shows that Lubrol WX can stimulate the activity of $(Na^+ + K^+)$-ATPase derived from the cerebral cortex of mice, and produces peak activation with 0.01% detergent. Ethanol at 0.05 and 0.5 M suppresses this activation. This inhibitory effect of ethanol gradually increases with the concentration of Lubrol WX. However, once the detergent concentration is about 0.01%, further increases do not enhance the ethanol effect. The activity of Mg^{2+}-dependent adenosine triphosphatase was not affected by Lubrol or ethanol at the concentrations used in this study. Other aliphatic alcohols, including

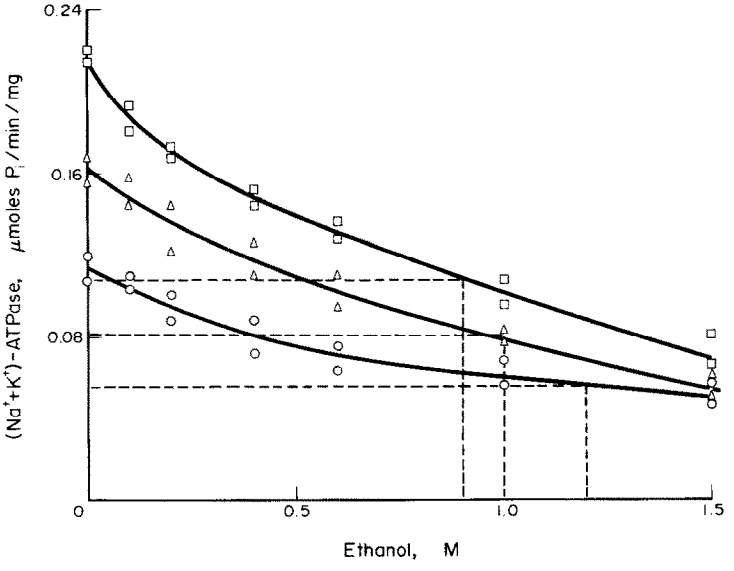

Fig. 2. Increases of ethanol sensitivity of $(Na^+ + K^+)$-ATPase by Lubrol WX in a crude preparation. Both the total ATPase and Mg^{2+}-ATPase assay mixtures [6] without (○) or with 0.005% (△) and 0.01% (□) Lubrol WX were incubated at 37° with the low-speed enzyme preparation (36.5 μg protein/assay tube) for 2 min. Ethanol was then added to various final concentrations. The assay mixtures were further incubated at 37° for 3 min, and the reactions were initiated by the addition of Tris–ATP. Each assay was duplicated. $(Na^+ + K^+)$-ATPase activity is the difference between the total ATPase and Mg^{2+}-ATPase activities [6].

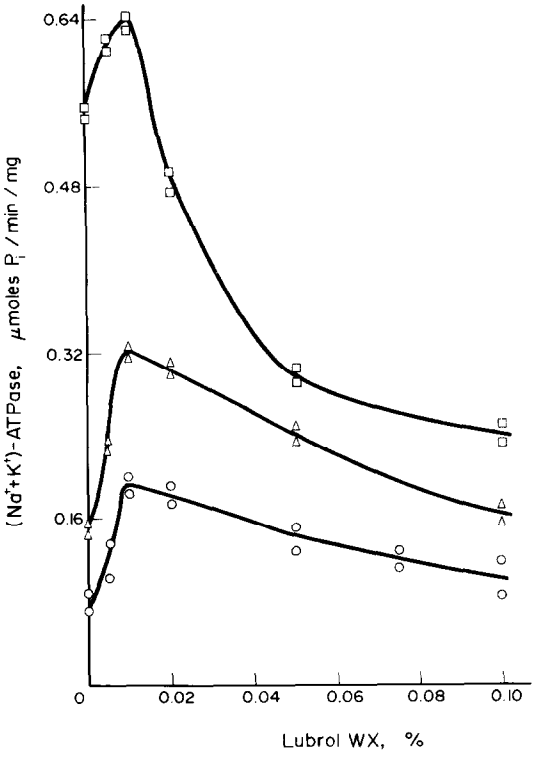

Fig. 3. Effect of Lubrol WX on the activity of $(Na^+ + K^+)$-ATPase purified to different extents. Both the total ATPase and Mg^{2+}-ATPase assay mixtures [6] with various concentrations of Lubrol WX were mixed with one of the following enzyme preparations: low-speed fraction (○), microsomal preparation (△) or NaI-treated enzyme (□). The mixtures were equilibrated at 37° for 5 min. The reactions were initiated by the addition of Tris–ATP, and the $(Na^+ + K^+)$-ATPase activity was ascertained [6]. Each assay was done in duplicate. The amount of protein in each assay tube was: low-speed fraction, 36.5 μg; microsomal preparation, 21.3 μg; and NaI-treated enzyme, 12.4 μg.

methanol, 1-propanol, 1-butanol and 1-pentanol, also produce this pattern of inhibitory effects on the activation of $(Na^+ + K^+)$-ATPase by Lubrol WX (data not shown). Figure 2 further illustrates that Lubrol WX can increase the sensitivity of $(Na^+ + K^+)$-ATPase to ethanol. In the presence of 0.005 and 0.01% detergent, the concentration of ethanol required to inhibit 50 per cent of the ATPase activity (I_{50}) was reduced from 1.2 M to 1.0 and 0.9 M respectively.

Since detergents have been thought to activate $(Na^+ + K^+)$-ATPase by exposing more enzyme sites [10, 11], the possibility exists that purification could make the enzyme more sensitive to ethanol inhibition but less sensitive to the stimulatory effect of Lubrol WX. As shown in Fig. 3, Lubrol WX has a biphasic effect on the $(Na^+ + K^+)$-ATPase activity of the low-speed and microsomal fractions from mouse brain, but has less effect on the NaI-treated preparation. Purification did increase the sensitivity of the enzyme to ethanol, as shown by the reduction in I_{50} (Table 1). However, the mass ratio of phospholipid to protein was not reduced, though there was a decrease in the amount of phospholipid per unit of ATPase.

Delipidization of the $(Na^+ + K^+)$-ATPase with deoxycholate reduced its ratio of phospholipid to protein, but did not make the remaining enzyme activity more sensitive to ethanol (Table 1). The I_{50} for the delipidized ATPase varied according to the ratio of added PS to protein; it was 1.5 ± 0.3 M when no exogenous phospholipids were present, but gradually decreased, and then increased again, with the concentration of PS (Table 2). Figure 4 further illustrates the dependency of the ethanol sensitivity of this reactivated ATPase on the ratio of PS to protein. In the absence of ethanol, the delipidized ATPase activity increases with the concentration of PS and reaches the maximum at a PS–protein ratio of 50 μmoles/mg. PC cannot reactivate the enzyme (data not shown). Ethanol suppresses the complete reactivation (Fig. 4). When the remaining ATPase activities in the presence of ethanol (0.40, 0.60 and 1.14 M) and at different PS–protein ratios were calculated as a percentage of their respective activities in the absence of ethanol, it was found that the enzyme activity was most sensitive to ethanol inhibition when it was half-reactivated at 12.5 μmoles PS P_i/mg protein. At the three ethanol concentrations tested, it was impossible to completely reverse the alcohol inhibition by increasing the ratio of PS to protein. At high PS concentrations, the enzyme

Table 1. Phospholipid content and ethanol sensitivity of various $(Na^+ + K^+)$-ATPase preparations*

Fraction	Specific activity (units/mg)	Phospholipid μmoles P_i / mg protein	Phospholipid μmoles P_i / unit ATPase	Ethanol I_{50} (M)
Low-speed fraction	0.123 ± 0.051	0.233 ± 0.085	1.894 ± 0.133	1.5 ± 0.3
Microsome	0.188 ± 0.063	0.313 ± 0.101†	1.665 ± 0.120†	1.3 ± 0.2†
NaI-fraction	0.496 ± 0.102	0.492 ± 0.120‡	0.992 ± 0.118§	1.1 ± 0.2§
Delipidized fraction	0.071 ± 0.013	0.198 ± 0.063†	2.789 ± 0.235§	1.5 ± 0.2†

* Each value is the mean ± S.E. of three duplicated determinations. The significance of the difference between each mean of the low-speed fraction and those of other enzyme preparations was evaluated by the *t*-test.

† Not significant.

‡ $P < 0.05$.

§ $P < 0.01$.

Table 2. Ethanol sensitivity of the delipidized $(Na^+ + K^+)$-ATPase at different phosphatidyl serine concentrations*

Vesicular phosphatidyl serine (mM P_i)	ATPase activity (units/mg)	Ethanol I_{50} (M)	N
0	0.071 ± 0.013	1.51 ± 0.31	4
1.0	0.213 ± 0.021	0.80 ± 0.12†	3
2.5	0.341 ± 0.027	0.93 ± 0.24‡	4
5.0	0.394 ± 0.025	1.12 ± 0.15§	7
7.5	0.372 ± 0.022	1.31 ± 0.19§	6

* Each value is the mean ± S.E. of three to seven duplicated determinations. The significance of the difference between the I_{50} obtained in the absence of exogenous PS and that obtained in the presence of each PS concentration was evaluated by the *t*-test.
† $P < 0.01$.
‡ < 0.05.
§ Not significant.

activity eventually levelled off before reaching the degree obtainable without ethanol.

DISCUSSION

While the inhibitory effect of ethanol on $(Na^+ + K^+)$-ATPase activity observed *in vitro* may not necessarily suggest that the enzyme is the site of alcohol action *in vivo*, this ATPase does provide an interesting model for studying the relative effect of ethanol on membrane lipids and proteins. The enzyme is membrane-bound, and probably extends to both sides of the lipid bilayer because it transports Na^+ out of, and K^+ into, the cell. Lipids which are closely associated with the enzyme, and necessary for its activity, can be considered 'essential lipids'. Those that are situated near the enzyme, but not required for its activity, can be considered 'bulk lipids'. Bulk lipids may hinder the access of the substrate and ligands to, and the release of products from, the ATPase. In this study, we observed that the delipidized $(Na^+ + K^+)$-ATPase from mouse brain can be reactivated with PS (Fig. 4), but not with PC. A similar result has been reported for the delipidized $(Na^+ + K^+)$-ATPase from the electric organ of *Electrophorus* [9] and rabbit kidney [12]. These data indicate that PS is an essential lipid for $(Na^+ + K^+)$-ATPase.

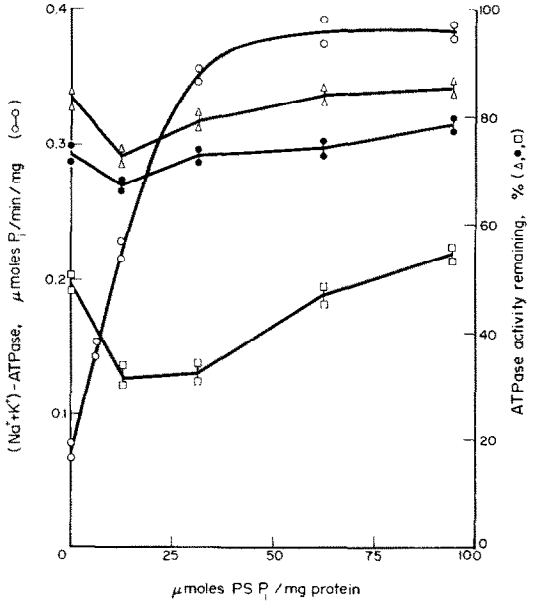

Fig. 4. Effect of phosphatidyl serine on the ethanol sensitivity of $(Na^+ + K^+)$-ATPase. $(Na^+ + K^+)$-ATPase assay mixtures [6] without (○) or with 0.4 M (△), 0.6 M (●) and 1.14 M (□) ethanol were mixed with the delipidized $(Na^+ + K^+)$-ATPase preparation (12.5 μg protein/assay tube) and various amounts of vesicular phosphatidyl serine. The mixtures were equilibrated at 37° for 5 min. The reactions were initiated by the addition of Tris–ATP, and $(Na^+ + K^+)$-ATPase activities were ascertained [6]. Each assay was duplicated. In the presence of ethanol, the $(Na^+ + K^+)$-ATPase activity at each PS/protein ratio was expressed as the percentage of its respective control without ethanol.

In cell-free preparations, $(Na^+ + K^+)$-ATPase is probably embedded in the lipid bilayer of vesicles [13], and some of the enzyme molecules may be concealed by bulk lipids. Lubrol WX may have activated the mouse brain ATPase (Fig. 1) by removing bulk lipids and contaminating proteins. Consequently, the enzyme would have better access to its substrate and cofactors, resulting in a higher specific activity (Fig. 3). The fact that purification does not reduce the lipid–protein ratio (Table 1) is most likely due to the simultaneous removal of contaminating proteins, such as Mg^{2+}-ATPase [11]. Since detergents at concentrations higher than their cmc can form mixed micelles with lipids or hydrophobic proteins [17] Lubrol WX at concentrations greater than 0.01%, which is near its cmc [11], could do so with $(Na^+ + K^+)$-ATPase, and therefore suppress its activity. This might explain the biphasic effect of the detergent on mouse brain ATPase (Fig. 1).

Removal of bulk lipids and contaminating proteins from the $(Na^+ + K^+)$-ATPase preparation, either by detergents or other means, may give ethanol a better access to interact directly with the enzyme. Consequently, the sensitivity of the enzyme to ethanol would be increased (Figs. 1 and 2 and Table 1). Ethanol may also interact with Lubrol WX. However, the interaction probably is weaker than it is with the $(Na^+ + K^+)$-ATPase, because increasing

the Lubrol WX concentration (up to 2%) does not reverse the ethanol inhibition of the ATPase activity (Fig. 1).

Delipidization by deoxycholate might strip both the bulk and essential lipids from $(Na^+ + K^+)$-ATPase [8, 12], and change the enzyme to a less active conformation. Addition of exogenous PS may then restore its conformation and activity. The sensitivity of the reactivated $(Na^+ + K^+)$-ATPase to ethanol varies with the ratio of PS to protein, and is most sensitive at 12.5 μmoles PS P_i/mg, when it is half-reactivated (Table 2 and Fig. 4). Further additions of PS gradually reduce this sensitivity, but cannot reverse it completely. This suggests that ethanol does not inhibit $(Na^+ + K^+)$-ATPase activity by interacting with the essential lipid (PS) alone, but may produce the inhibition by interacting with the protein moiety of the ATPase, especially when the enzyme assumes favorable conformations following the binding of PS.

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