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# EFFECTS OF ETHANOL ON OUABAIN INHIBITION OF MOUSE BRAIN ($Na^+$,$K^+$)ATPase ACTIVITY

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**Abstract**—Plots of ouabain inhibition of mouse cerebral cortical ($Na^+$,$K^+$)ATPase activity fitted a two-site model significantly better than a one-site model, consistent with the presence of two forms of the enzyme with different affinities for ouabain. The fraction of enzyme activity with high affinity for ouabain (HAO: $K_i$ = 500 nM), suggested to be localized neuronally, constituted the major portion (60–70%) of activity. Ouabain inhibition of both components of enzyme activity was reduced as KCl concentrations were increased. *In vitro*, only high concentrations of ethanol affected ($Na^+$,$K^+$)ATPase activity and ouabain inhibition of activity. Ethanol (500 mM) selectively reduced the activity, and increased the sensitivity to ouabain inhibition, of the HAO component, with no significant effect on the low-affinity (LAO) component. On the other hand, following chronic treatment of mice with ethanol *in vivo*, in a paradigm that produced tolerance and physical dependence, the sensitivity to ouabain of the HAO form of the enzyme was selectively increased. The relative proportions, and the activities of the HAO and LAO components, were not altered. The effects of ethanol, added *in vitro*, on the HAO component were decreased in ethanol-tolerant animals. The selective effect of chronic ethanol ingestion on ($Na^+$,$K^+$)ATPase activity indicates the specificity of action of ethanol in the CNS.

In brain, the enzyme sodium-potassium-transporting adenosine triphosphatase [EC 3.6.1.37, ($Na^+$,$K^+$)ATPase] appears to exist in two forms, which have structural and kinetic differences [1, 2], including different affinities for the inhibitor of enzyme activity, ouabain [1, 2]. It has been suggested that the form of the enzyme with high affinity for ouabain (HAO§) is specific for neuronal rather than glial tissue [2, 3], and the activity of this form or fraction of the enzyme appears to be selectively regulated by neurotransmitters [4].

The mechanism by which ouabain inhibits enzyme activity is still under investigation [5]. The ($Na^+$,$K^+$)ATPase reaction involves a series of interconversions between two main enzyme conformations: $E_1$, with high affinity for ATP and $Na^+$, and $E_2$, with high affinity for $K^+$ [6]. Ouabain binds preferentially to the phosphorylated $E_2$ conformation ($E_2P$) [7, 8]. The amount of enzyme in the $E_2P$ conformation [9], and the sensitivity of enzyme activity to ouabain inhibition [10, 11], are both increased by increases in membrane fluidity, and decreased by interaction of $K^+$ with the ($Na^+$,$K^+$)ATPase regulatory site.

Ethanol, *in vitro*, at high concentrations, inhibits the activity of brain ($Na^+$,$K^+$)ATPase [12–15] and decreases the affinity of the enzyme for $K^+$ [12, 14–17]. These effects may result, in part, from ethanol-induced perturbation of neuronal membrane lipid properties [17, 18], which could also affect affinity for ouabain. A previous report suggested that the form of ($Na^+$,$K^+$)ATPase with high affinity for ouabain was more sensitive to inhibition by ethanol [19].

After chronic *in vivo* treatment of animals with ethanol, such that they display tolerance to and physical dependence on ethanol, the affinity of ($Na^+$,$K^+$)ATPase for $K^+$ is reduced [17, 18], and enzyme activity shows resistance to the *in vitro* inhibitory effect of ethanol [17, 20, 21]. The resistance to inhibition has been postulated to result from ethanol-induced alterations in the properties of neuronal lipids surrounding the enzyme [17, 20]. However, another possible explanation, if the two structural forms of ($Na^+$,$K^+$)ATPase have different sensitivities to inhibition by ethanol [19], is that a change in the relative proportion of the two forms occurs after chronic ingestion of ethanol. We have, therefore, in the present study, investigated the acute *in vitro*, as well as the chronic *in vivo*, effects of ethanol on ouabain affinity and the activities of the two components of mouse brain ($Na^+$,$K^+$)ATPase activity.

## MATERIALS AND METHODS

*Chronic ethanol administration.* Male C57B1 mice (22–25 g) (NCI, Frederick, MD) were housed six per

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§ Abbreviations: HAO, component of ($Na^+$,$K^+$)ATPase activity with high affinity for ouabain; LAO, component of ($Na^+$,$K^+$)ATPase activity with low affinity for ouabain.



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cage in our laboratories for at least 7 days, under conditions of constant temperature and lighting (12 hr light–dark cycle), and with *ad lib.* access to rodent chow and water, before initiating experiments. For chronic ethanol ingestion, mice were housed individually and fed either a control liquid diet or an ethanol-containing liquid diet. Details of this method have been published previously [22]. The quantity of sucrose-containing diet fed daily to control animals was made equal to the average amount of diet consumed by the ethanol-fed animals. After 7 days, all animals were again given the control diet (ethanol withdrawal). Ethanol-consuming animals were monitored over a period of 12 hr for signs of an alcohol abstinence syndrome (tremors, handling-induced seizures) [22], and both experimental and control animals were fed the control diet until they were killed at 24 hr after withdrawal. In some experiments, certain animals, which were not used in the biochemical studies, were tested for tolerance to the hypnotic effects of an injection of ethanol by previously described methods [22], at 24 hr after withdrawal. $(Na^+,K^+)$ATPase in tissue of naive (chow-fed), control (liquid diet-fed) and ethanol-fed mice was assayed simultaneously.

*Preparation of tissue.* Mice were decapitated, brains were removed quickly, and the cerebral cortex was dissected at 4°. Pooled tissue from three mice per experiment was homogenized in 20 vol. of ice-cold 50 mM imidazole buffer containing 2 mM Tris, 2 mM EDTA and 270 mM sucrose (pH 7.2), and centrifuged at 600 *g* for 10 min. The supernatant fraction was centrifuged at 30,000 *g* for 20 min to produce the crude synaptosomal pellet. The pellet was resuspended in 10 mM imidazole buffer containing 1 mM Tris and 1 mM EDTA (pH 7.2), and centrifuged at 30,000 *g* for 20 min. This process was repeated three times. The washed crude synaptosomal preparation was resuspended in twice the volume of homogenate of 50 mM Tris–HCl (pH 7.4), and the suspension was used immediately or stored overnight at −70°.

*Ouabain inhibition of $(Na^+,K^+)$ATPase activity.* $(Na^+,K^+)$ATPase activity was determined by the method of Atterwill *et al.* [23]. The synaptosomal suspension (100 μg protein [24]) was incubated in a total volume of 1.0 ml containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM or 20 mM KCl, 100 mM NaCl, 5 mM Tris–ATP, an ATP-regenerating system (4 mM phosphoenolpyruvate and 3 units pyruvate kinase), and a series of twenty-two concentrations of ouabain ranging from $10^{-9}$ to $10^{-2}$ M. Tubes were preincubated at 30° for 60 min, a time course necessary for maximal inhibition of $(Na^+,K^+)$ATPase activity by ouabain (data not shown), prior to addition of [$\gamma^{32}$P]ATP (500,000 cpm) and incubation for a further 30 min. The reaction was terminated by the addition of freshly made ice-cold 12% (w/v) activated charcoal in 0.8% (w/v) sodium dodecyl sulfate (SDS) solution. Tubes were mixed and left on ice for at least 10 min and then centrifuged at 22,000 *g* for 10 min. The supernatant fraction was decanted into pre-cooled tubes. The remaining charcoal pellet was dispersed in 1.0 ml of ice-cold distilled water and centrifuged as described above. The two supernatant fractions were pooled and centrifuged to remove any remaining traces of charcoal. Aliquots of the supernatant (1.0 ml) were added to ACS scintillant (Amersham Radiochemicals, Arlington Heights, IL), and radioactivity was quantitated on an LKB Rackbeta III liquid scintillation spectrometer. $(Na^+,K^+)$ATPase activity was calculated as the difference in the rate of reaction in the presence and absence of $Na^+$ and $K^+$. Blanks that did not contain the enzyme preparation were used to determine the efficiency of the charcoal to remove labeled ATP. In all instances, 95–98% of the substrate was removed by this process. [$^{32}$P]Orthophosphoric acid was used to estimate $^{32}P_i$ recovery (80–90%), and all values were corrected both for the blank values and for $^{32}P_i$ recovery.

*Measurement of [$^3$H]digitoxin binding.* Digitoxin binding was measured using a cell harvester (M-24R, Brandel, Gaithersburg, MD). The assay conditions were identical to those described above for measurement of $(Na^+,K^+)$ATPase activity. [$^3$H]Digitoxin (1 nM; 13.8 Ci/mmol) replaced [$\gamma$-$^{32}$P]ATP, and tubes were incubated at 30° for various time periods. The assays were terminated by filtration of tissue suspensions under vacuum through Whatman GF/B glass fiber filters. The filters were washed with three 5-ml volumes of ice-cold 50 mM Tris–HCl (pH 7.4) and placed in ACS scintillation fluid for determination of bound radioactivity (counting efficiency approximately 40%). Specific ligand binding was defined as the difference between binding in the absence and presence of 6 mM ouabain. Non-specific binding was about 10% of total binding.

*Addition of ethanol.* In both enzyme assays and binding experiments, ethanol solutions were added to the reaction mixture at the beginning of the preincubation period, and tubes were capped throughout the assay.

*Analysis of data.* The data for inhibition of $(Na^+,K^+)$ATPase activity by ouabain were fitted to the following rate equations [4]:

$$V = 1-[HS/(K_{i_1} + S)] \quad (1)$$

$$V = 1-[HS/(K_{i_1} +S) + [(1-H)/(K_{i_2} +S)]] \quad (2)$$

Equation 1 represents a one-site model for inhibitor (ouabain) action, and Equation 2 represents a two-site model. $V$ is the observed reaction rate as a fraction of the rate in the absence of ouabain: $H$ is the fraction of enzyme activity which is inhibited by 50% at the ouabain concentration equal to $K_{i_1}$ (i.e. "HAO" see below); $(1-H)$ is the fraction of enzyme activity which is inhibited by 50% at the ouabain concentration equal to $K_{i_2}$ (i.e. "LAO"); and $S$ is the ouabain concentration. The values for $K_{i_1}$, $K_{i_2}$ and $H$ were obtained by means of non-linear weighted least squares minimization using the program PENNZYME [25]. This program was obtained from the Share Program Library Agency (no. 360D.13.2.004; Research Triangle Park, NC) and was adapted for use on a PDP-11 (N. Shioura, University of Illinois at Chicago) or a VAX 11/750 (E. Lamoreaux, NIAAA, Rockville, MD). The program includes statistical tests for the reliability of the derived parameters and for error due to fitting the data to a poor model. The F-statistic provided by the

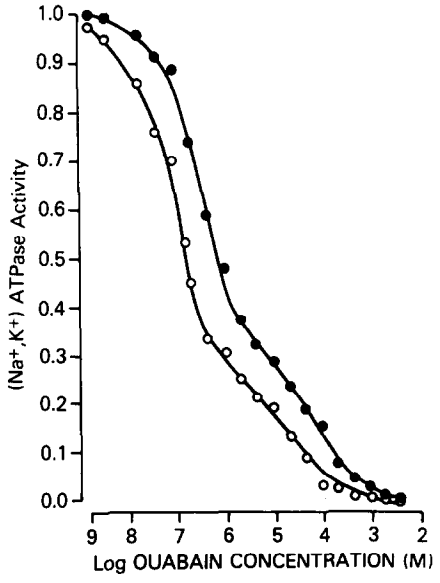


Fig. 1. Inhibition by ouabain of (Na⁺,K⁺)ATPase activity in cerebral cortical membranes from naive (chow-fed) mice. (Na⁺,K⁺)ATPase activity was assayed as described in the text, in the presence of either 5 mM (○) or 20 mM KCl (●). Enzymatic activity at each concentration of ouabain is expressed as a fraction of that in the absence of ouabain. The values shown represent the mean of three separate experiments, and standard deviations average 4% (not shown).

in the trial rate law and the number of velocity measurements in a curve respectively.

For comparisons between groups, Student's *t*-test was used, with $P < 0.05$ chosen as significant.

## RESULTS

*Ouabain inhibition of (Na⁺,K⁺)ATPase activity in naive (chow-fed) mice.* (Na⁺,K⁺)ATPase activity was inhibited by ouabain in a biphasic manner (Fig. 1). The inhibition data fitted a two-site model significantly ($P < 0.001$) better than a one-site model. In naive (chow-fed) mice, the high-affinity (HAO) component constituted a greater fraction of total (Na⁺,K⁺)ATPase activity (60–70%) than the low-affinity component (LAO) (Table 1). The values of the apparent ouabain inhibition constants were influenced by the concentration of KCl (Table 1). Decreasing the concentration of KCl in the incubation mixture from 20 to 5 mM caused significant *increases* in the sensitivity to ouabain inhibition of the HAO (by about 3-fold) and LAO (by 2-fold) components of (Na⁺,K⁺)ATPase activity.

In the presence of 500 mM ethanol *in vitro*, ouabain inhibition curves of cortical (Na⁺,K⁺)-ATPase activity from naive (chow-fed) mice consisted of two components, with high and low affinity for ouabain, as had been observed in the absence of ethanol. However, ethanol significantly lowered the apparent inhibition constant for ouabain of the HAO

Table 1. Effect of ethanol on inhibition of (Na⁺,K⁺)ATPase activity by ouabain in cerebral cortical membranes from naive (chow-fed) mice

| | Ouabain inhibition constants and proportion of HAO and LAO components | | | |
|---|---|---|---|---|
| | Ouabain $K_{i_1}$ (nM) | % HAO | Ouabain $K_{i_2}$ (μM) | % LAO |
| 20 mM KCl (N = 4) | | | | |
| No addition | 535 ± 91 | 72 ± 8 | 116 ± 21 | 29 ± 6 |
| 500 mM Ethanol | 221 ± 55* | 68 ± 10 | 122 ± 25 | 32 ± 5 |
| % Change | 58.7 ± 12.3 | NS† | NS | NS |
| 5 mM KCl (N = 5) | | | | |
| No addition | 156 ± 26‡ | 65 ± 5 | 50 ± 14‡ | 35 ± 3 |
| 500 mM Ethanol | 62 ± 19‡,§ | 61 ± 6 | 54 ± 15‡ | 45 ± 4 |
| % Change | 39.7 ± 9.4 | NS | NS | NS |

(Na⁺,K⁺)ATPase activity was assayed as described in the text in the presence of either 5 mM or 20 mM KCl. Ouabain inhibition constants, and the proportion of HAO and LAO components, were calculated using the PENNZYME program. Values represent mean ± SD, from the number of experiments shown in parentheses.

* $P < 0.01$ compared to respective control (no addition) values (Student's *t*-test, df = 6).

† Not significant.

‡ $P < 0.001$ compared to corresponding values at 20 mM KCl (Student's *t*-test, df = 7).

§ $P < 0.001$ compared to respective control (no addition) values (Student's *t*-test, df = 8).

program takes into account the residual error ($\sigma$), which reflects experimental errors in the velocity measurements ($\sigma^*$), as well as bias resulting from fitting an incorrect model to the data ($F = \sigma^2/(\sigma^*)^2$) (see Ref. 25). If $F$ exceeded its tabulated value for $n - p$ (numerator) and $n - 1$ (denominator) degrees of freedom at the 0.05 level, the model was rejected. $p$ and $n$ represent the number of kinetic parameters

component ($K_{i_1}$) at either concentration of KCl, (Table 1). On the other hand, the affinity of the LAO component for ouabain was not altered significantly by ethanol (Table 1).

*Effect of chronic ethanol ingestion on ouabain inhibition of (NA⁺,K⁺)ATPase activity.* The chronic ethanol regimen produced tolerance to and physical dependence on ethanol. In a typical experiment, the

Table 2. Effect of chronic ethanol ingestion on ouabain inhibition of $(Na^+,K^+)$ATPase activity in mouse cerebral cortical membranes

| | 20 mM KCl | | | 5 mM KCl | | |
|---|---|---|---|---|---|---|
| | Ouabain $K_{i_1}$ (nM) | Ouabain $K_{i_2}$ ($\mu$M) | % HAO | Ouabain $K_{i_1}$ (nM) | Ouabain $K_{i_2}$ ($\mu$M) | % HAO |
| Control | | | | | | |
| No addition | 522 ± 65 (9) | 119 ± 8 (9) | 68 ± 7 (9) | 145 ± 15* (6) | 46 ± 6* (6) | 60 ± 7 (6) |
| 500 mM Ethanol | 205 ± 56† (7) | 98 ± 25 (7) | 64 ± 7 (7) | 75 ± 11† (4) | 42 ± 8 (4) | 65 ± 9 (4) |
| Ethanol-withdrawn | | | | | | |
| No addition | 254 ± 45‡ (9) | 121 ± 23 (9) | 69 ± 5 (9) | 118 ± 13*,‡ (6) | 48 ± 7* (6) | 75 ± 4 (6) |
| 500 mM Ethanol | 198 ± 35 (4) | 120 ± 36 (4) | 67 ± 8 (4) | 97 ± 5 (4) | 47 ± 7 (4) | 72 ± 8 (4) |

$(Na^+,K^+)$ATPase activity was assayed as described in the text in the presence of either 5 mM or 20 mM KCl. Ouabain inhibition constants, and the proportion of the HAO component, were calculated using the PENNZYME program. Animals were fed ethanol in a liquid diet, or control diet, and enzyme activity was measured at 24 hr after withdrawal from ethanol. Values represent mean ± SD from the number of experiments shown in parentheses. Degrees of freedom for statistical analysis = $n_1 + n_2 - 2$.

* $P < 0.001$ compared to corresponding ouabain $K_i$ at 20 mM KCl (Student's $t$-test).

† $P < 0.001$ compared to "no addition" value (Student's $t$-test).

‡ $P < 0.001$ compared to "no addition" value of membranes from animals fed control liquid diet (Student's $t$-test).

duration of loss of righting reflex after a dose of 3.2 g/kg of ethanol was (mean ± SEM, N = 5 per group): controls, 34.6 ± 9.1 min; ethanol-withdrawn, 3.3 ± 6.4. Overt withdrawal signs, including tremors and seizures, measured as described previously [22], had dissipated at the time of sacrifice (24 hr after withdrawal). The properties of $(Na^+,K^+)$ATPase in cerebral cortical membranes from animals fed the control liquid diet were not significantly different from those of naive (chow-fed) mice, with respect to the activities and proportions of the two components, affinities of the two components for ouabain, and response to 500 mM ethanol *in vitro* (compare Tables 1 and 2, and compare Table 3 and Fig. 2). Ouabain inhibition curves of cerebral cortical $(Na^+,K^+)$ATPase activity in membranes from the ethanol-withdrawn mice still consisted of two components, with high and low affinity for ouabain. After chronic ethanol feeding, however, there was a statistically significant decrease in the ouabain inhibition constant ($K_{i_1}$) of the HAO component, in the absence of added ethanol *in vitro* (Table 2). The magnitude of the change was greater at the higher $K^+$ concentration (20 vs 5 mM). As in mice fed control liquid diet or lab chow, lowering the $K^+$ concentration caused increases in the affinity for ouabain of both HAO and LAO components in ethanol-withdrawn mice. However, the inhibition constant of the HAO component ($K_{i_1}$) in ethanol-withdrawn animals was less sensitive to changes in $K^+$ concentration than that of controls. Thus, the decrease in $K^+$ concentration caused a 2.2-fold increase in sensitivity to ouabain inhibition of the HAO component in membranes from ethanol-treated mice, compared to a 3.6-fold increase in controls or naive (chow-fed) mice (Tables 1 and 2).

Addition of 500 mM ethanol *in vitro* to membranes from ethanol-withdrawn mice no longer elicited a significant decrease in $K_{i_1}$ for ouabain, compared to the effect in cerebral cortical membranes from mice that had received control liquid diet (Table 2) or naive (chow-fed) mice (Table 1). The ouabain inhibition constant ($K_{i_2}$) of the low-affinity component, and its sensitivity to $K^+$, were not altered significantly following chronic administration of ethanol to mice (Table 2). In addition, ethanol *in vitro* had no significant effect on ($K_{i_2}$) in any of the groups of animals (Tables 1 and 2).

*Effect of ethanol* in vitro *and of chronic ethanol ingestion on* $(Na^+,K^+)$*ATPase activity.* A high concentration of ethanol *in vitro* inhibited $(Na^+,K^+)$ATPase activity in naive (chow-fed) (Table 3) and control (liquid diet-fed) mice (Fig. 2). As reported previously [12, 14–17], the inhibitory effect of ethanol was significantly greater at the low KCl concentration. This difference was seen in naive (chow-fed) mice (Table 3) as well as in liquid diet controls (Fig. 2). Thus, in the presence of 5 mM KCl, 500 mM ethanol inhibited total $(Na^+,K^+)$ATPase activity by about 30% compared to 18% in the presence of 20 mM KCl. However, this inhibitory effect of ethanol was exclusively localized to the HAO fraction, with no significant inhibition of the LAO fraction in the chow-fed and control animals (Table 3 and Fig. 2).

Although chronic ethanol administration did not alter significantly the total activities of the HAO or LAO components at either KCl concentration (Fig. 2), this regimen resulted in cerebral cortical membranes in which, at 5 mM KCl, the HAO component of $(Na^+,K^+)$ATPase activity showed a significant decrease in sensitivity to inhibition by ethanol *in vitro*, compared to either control (liquid diet-fed) (Fig. 2) or naive (chow-fed) mice (Table 3).

*Effect of ethanol on specific binding of* [$^3$H]*digitoxin.* The specific binding of 1 nM [$^3$H]digitoxin was time dependent, reaching maximal levels in about 60 min (Fig. 3). The values of the apparent rate constant for binding ($k_{ob}$) [26] were 0.043 ±0.003 $min^{-1}$ and 0.029 ± 0.002 $min^{-1}$ at 5 mM and 20 mM $K^+$, respectively (mean ± SD; N = 3). Thus, lowering the concentration of KCl in the incubation mixture caused a significant ($P < 0.05$) increase (48%) in the rate constant for digitoxin binding. In addition, the amount of [$^3$H]digitoxin bound at 60 min was significantly greater (by 35%) at the low

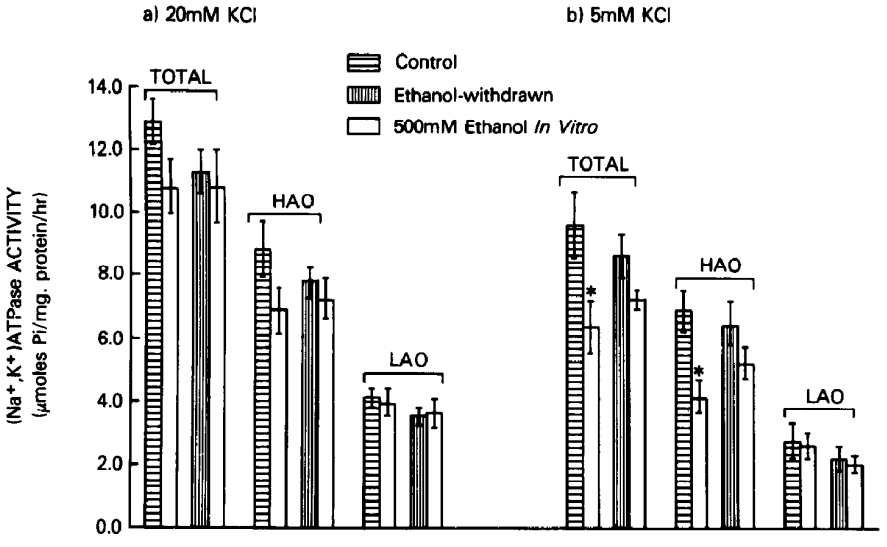


Fig. 2. Effects of ethanol on (Na⁺,K⁺)ATPase activities in cerebral cortical membranes from C57B/1 mice. Animals were fed ethanol in a liquid diet, or control diet, and (Na⁺,K⁺)ATPase activity was measured at 24 hr after withdrawal, in the absence (shaded) and presence (not shaded) of 500 mM ethanol (see text). The HAO and LAO activities of control mice (▤) and ethanol-withdrawn mice (▥) were calculated using the PENNZYME program. Values represent the mean ± SD of at least four separate experiments.

Table 3. Effect of ethanol on (Na⁺,K⁺)ATPase activity in cerebral cortical membranes from naive (chow-fed) mice

| | Enzyme activity (μmole $P_i$/mg protein/hr) | | |
|---|---|---|---|
| | Total | HAO | LAO |
| 20 mM KCl (N = 4) | | | |
| No addition | 12.41 ± 1.76 | 8.85 ± 0.49 | 3.56 ± 0.76 |
| 500 mM Ethanol | 10.10 ± 1.59 | 6.84 ± 0.73 | 3.26 ± 0.49 |
| % Change | NS* | NS | NS |
| 5 mM KCl (N = 5) | | | |
| No addition | 11.41 ± 0.91 | 7.43 ± 0.45 | 3.98 ± 0.35 |
| 500 mM Ethanol | 8.01 ± 0.70† | 4.89 ± 0.45† | 3.64 ± 0.36 |
| % Change | 29.8 ± 4.5 | 34.2 ± 6.5 | NS |

(Na⁺,K⁺)ATPase activity was assayed as described in the text in the presence of either 5 mM or 20 mM KCl. The activities of the HAO and LAO components were calculated using the PENNZYME program. Values represent mean ± SD, from the number of experiments shown in parentheses.

* Not significant.

† $P < 0.05$ compared to respective control ("no addition") values (Student's *t*-test, df = 8).

concentration of $K^+$ (Fig. 3). In the presence of 500 mM ethanol, the value of the rate constants increased to $0.058 \pm 0.004\ min^{-1}$ and $0.038 \pm 0.002\ min^{-1}$ at 5 mM and 20 mM $K^+$ respectively. Ethanol also increased the specific binding of [³H]digitoxin in the presence of 5 mM and 20 mM KCl (Fig. 3).

## DISCUSSION

Our results are consistent with the presence, in mouse brain, of two forms of (Na⁺,K⁺)ATPase activity with different affinities for ouabain [1–3]. At a high concentration of KCl (20 mM), the sensitivity to ouabain inhibition of both forms of (Na⁺,K⁺)ATPase activity was reduced, supporting the finding that $K^+$ decreases the affinity of the enzyme for ouabain [27, 28]. This result was also supported by digitoxin binding experiments in which binding was reduced at 20 mM KCl, as compared to 5 mM KCl (Fig. 3).

Chronic ingestion of ethanol, which produced tolerance and physical dependence, resulted in a selective increase in sensitivity to ouabain inhibition of the HAO component of brain (Na⁺,K⁺)ATPase activity, which has been postulated to be localized neuronally [2, 3]. This change was not a result of nutritional factors, since the characteristics of enzyme activity were similar in naive (chow-fed) and control (liquid diet-fed) mice. Other factors, however, could underlie the change in properties of the neuronal enzyme. They include changes in affin-

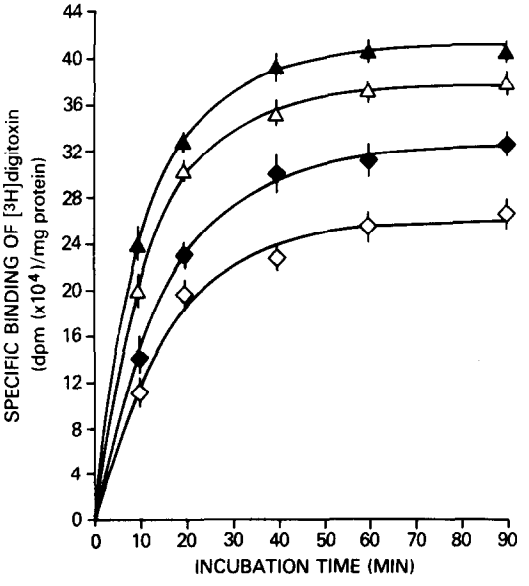


Fig. 3. Effects of 5 mM (△, ▲) and 20 mM (◇,◆) KCl on the time course of specific binding of 1 nM [$^3$H]digitoxin to cerebral cortical membranes from naive (chow-fed) mice in the presence (closed) and absence (open) of 500 mM ethanol. The assay conditions were as described in the text. Each point is the mean ± SD of at least three separate experiments; in each experiment, assays were in duplicate.

ity of the enzyme for $K^+$ [17, 18], and alterations in membrane fluidity of "boundary" lipids of the HAO component [17, 20]. Rangaraj *et al.* [20] and Levental and Tabakoff [17] reported a decrease in affinity for $K^+$ of $(Na^+,K^+)$ATPase in brains of ethanol-tolerant mice and rats. On the other hand, Swann [29] recently reported an *increase* in affinity for $K^+$ of $K^+$-*p*-nitrophenyl phosphatase in brains of ethanol-tolerant rats. Since, as shown in the present study, $K^+$ decreases the sensitivity of $(Na^+,K^+)$ATPase activity to inhibition by ouabain, the *increase* in sensitivity of the HAO component of the enzyme to inhibition by ouabain, following chronic ingestion of ethanol, is consistent with a decrease in the affinity for $K^+$ of $(Na^+,K^+)$ATPase, rather than an increase. However, if the effects of chronic ethanol ingestion were due solely to a decrease in affinity for $K^+$, one would expect a greater change to be evident at the $K^+$ concentration closer to the $K_m$ for $K^+$ (5 mM), as compared to 20 mM $K^+$. In fact, the change in $K_{i_1}$ for ouabain in ethanol-withdrawn animals was greater at 20 mM than at 5 mM $K^+$, suggesting the involvement of other factors.

Phospholipid-depleted brain membranes show a requirement for acidic phospholipids in restoration of $(Na^+,K^+)$ATPase activity [30], and in the rate of ouabain binding to the HAO component [31]. Although the properties of brain membranes from ethanol-treated animals have been reported to be different from those of controls [32, 33], complex and variable lipid changes have been observed [34]. However, there have been reports of increases in acidic phospholipids in brain membranes of ethanol-treated animals [35, 36]. Such changes in membrane phospholipid composition may occur within restricted areas of the cell membrane, making them difficult to detect when the composition of bulk lipids is determined [34]. Nevertheless, these changes could directly alter enzyme characteristics, or could affect the properties of the membrane in limited regions. Thus, although the change in ouabain affinity of the HAO fraction of $(Na^+,K^+)$ATPase probably does not affect *in vivo* enzyme activity, this change may be an indicator of selective changes in the lipid composition and physical properties of specific areas of the neuronal membrane, as a result of chronic ethanol ingestion.

Further evidence for this hypothesis is the finding that the transition temperature of $(Na^+,K^+)$ATPase was decreased in brains of ethanol-withdrawn animals [17, 20], indicative of a change in the properties of the membrane lipids immediately surrounding the enzyme. Whether the change in transition temperature was restricted to the HAO component was not determined in previous studies [17, 20]. However, ethanol *in vitro* was reported to selectively change the transition temperature of this component of enzyme activity [37].

In addition to changes in ouabain affinity after chronic ethanol ingestion, we found that membranes from ethanol-withdrawn mice showed resistance to the effect of a high concentration of ethanol on both the activity and sensitivity to ouabain inhibition of the HAO form of the enzyme i.e. tolerance developed to these *in vitro* effects of ethanol. However, neither the relative proportions nor the basal activities of the HAO and LAO components were altered significantly by chronic ethanol ingestion, indicating that this treatment did not induce an interconversion between the two structural forms of $(Na^+,K^+)$-ATPase activity. Thus, the reduced inhibitory effect of ethanol *in vitro* on total $(Na^+,K^+)$ATPase activity in membranes from ethanol-tolerant animals [17, 20, 21] is apparently not a result of an increased proportion of the LAO component, but of a change in properties of the HAO component.

In the present work, as in earlier studies [17–19, 38], a high concentration of ethanol *in vitro* was necessary to produce significant inhibition of $(Na^+,K^+)$ATPase activity, as well as changes in affinity for ouabain. Such ethanol concentrations could not be tolerated *in vivo*, and it is not suggested that the acute, *in vitro* effects of ethanol on $(Na^+,K^+)$ATPase activity necessarily contribute to the acute, *in vivo* physiological effects of ethanol. However, the *in vitro* response of $(Na^+,K^+)$ATPase to ethanol makes this enzyme useful as a probe that reflects changes in the properties of specific areas of the neuronal membrane, or in the characteristics of membrane-bound enzymes, that are produced by chronic ethanol ingestion [17, 20]. For example, it has been suggested that ethanol *in vitro* decreases the affinity of $(Na^+,K^+)$ATPase regulatory sites for $K^+$ [9, 17, 20], and also stabilizes the $E_2P$ conformation [39]. Both of these effects have been suggested to be mediated by changes in the fluidity of lipids immediately surrounding the enzyme [39]. Our results are consistent with the hypothesis that, after chronic, *in vivo*, ethanol ingestion, the properties of the lipids surrounding the HAO form of brain $(Na^+,K^+)$ATPase, or the properties of the protein

itself [38], are selectively altered, resulting in changes in ouabain sensitivity, as well as a reduced response to the effects of ethanol added *in vitro*. The selective sensitivity of the HAO form of the enzyme, in contrast to the LAO form, supports the postulate that these enzymes are separate and independent entities [1–3], and indicates the specificity of the chronic effects of ethanol in the CNS.

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## REFERENCES

1. M. J. Marks and N. W. Seeds, *Life Sci.* **23**, 2735 (1978).
2. K. J. Sweadner, *J. biol. Chem.* **254**, 6060 (1979).
3. S. C. Specht, *Biochem. biophys. Res. Commun.* **121**, 208 (1984).
4. A. C. Swann, *J. Pharmac. exp. Ther.* **228**, 304 (1984).
5. O. Hansen, *Pharmac. Rev.* **36**, 143 (1984).
6. J. L. Dahl and L. E. Hokin, *A. Rev. Biochem.* **43**, 327 (1974).
7. R. L. Post, S. Kume, T. Tobin, B. Orcutt and A. K. Sen, *J. gen. Physiol.* **54**, 306s (1969).
8. A. Yoda and S. Yoda, *Molec. Pharmac.* **22**, 700 (1982).
9. A. C. Swann, *J. biol. Chem.* **258**, 11780 (1983).
10. J. S. Charnock, L. P. Simonson and A. Almeida, *Biochim. biophys. Acta* **465**, 77 (1977).
11. L. K. Kane, C. M. Anner, E. T. Wallick, M. V. Ray and A. Schwartz, *Biochem. Pharmac.* **27**, 225 (1978).
12. Y. Israel, H. Kalant and I. Laufer, *Biochem. Pharmac.* **14**, 1803 (1965).
13. Y. Israel, H. Kalant and A. E. LeBlanc, *Biochem. J.* **100**, 27 (1966).
14. Y. Israel and I. Salazar, *Archs. Biochem. Biophys.* **122**, 310 (1967).
15. A. Y. Sun and T. Samorajski, *J. Neurochem.* **17**, 1365 (1970).
16. H. Kalant, N. Woo and L. Endrenyi, *Biochem. Pharmac.* **27**, 1353 (1978).
17. M. Levental and B. Tabakoff, *J. Pharmac. exp. Ther.* **212**, 315 (1980).
18. N. Rangaraj and H. Kalant, *Biochem. Pharmac.* **27**, 1139 (1978).
19. M. J. Marks, A. Smolen and A. C. Collins, *Alcoholism: Clin. expl. Res.* **8**, 390 (1984).
20. N. Rangaraj and H. Kalant, *J. Pharmac. exp. Ther.* **223**, 536 (1982).
21. F. Beaugè, C. Fleuret-Balter, J. Nordmann and R. Nordmann, *Alcoholism: Clin. expl. Res.* **8**, 167 (1984).
22. R. F. Ritzmann and B. Tabakoff, *J. Pharmac. exp. Ther.* **199**, 158 (1976).
23. C. K. Atterwill, V. J. Cunningham and R. Balaz, *J. Neurochem.* **43**, 8 (1984).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. M. C. Kohn, L. E. Menten and D. Garfinkel, *Comput. biomed. Res.* **12**, 461 (1979).
26. J. P. Bennett Jr., in *Neurotransmitter Receptor Binding* (Eds. H. I. Yamamura, S. J. Enna and M. J. Kuhar), pp. 57–90. Raven Press, New York (1978).
27. P. F. Baker and J. S. Willis, *Nature, Lond.* **226**, 521 (1970).
28. Y. R. Choi and T. Akera, *Biochim. biophys. Acta* **481**, 648 (1977).
29. A. C. Swann, *J. Pharmac. exp. Ther.* **232**, 475 (1985).
30. J. S. Charnock, A. F. Almeida and R. To, *Archs Biochem. Biophys.* **167**, 480 (1975).
31. K. Taniguchi and S. Iida, *Molec. Pharmac.* **9**, 350 (1973).
32. J. H. Chin and D. B. Goldstein, *Science* **196**, 684 (1977).
33. R. A. Harris and F. Schroeder, *Molec. Pharmac.* **20**, 128 (1981).
34. P. L. Hoffman and B. Tabakoff, in *Alcohol and the Brain: Chronic Effects* (Eds. R. E. Tarter and D. H. van Thiel), pp. 19–68. Plenum Medical Book Co., New York (1985).
35. R. C. Aloia, J. Paxton, J. S. Daviau, V. O. Gelb, W. Mlekusch, W. Truppe, J. A. Meyer and F. S. Brauer, *Life Sci.* **36**, 1003 (1985).
36. G. Y. Sun, H. M. Huang, D. Z. Lee and A. Y. Sun, *Life Sci.* **35**, 2127 (1984).
37. A. C. Collins, A. Smolen, A. L. Wayman and M. J. Marks, *Alcohol* **1**, 237 (1984).
38. P. T. Nhamburo, B. P. Salafsky, P. L. Hoffman and B. Tabakoff, *Biochem. Pharmac.* **35**, 1987 (1986).
39. H. Kalant and N. Rangaraj, *Eur. J. Pharmac.* **70**, 157 (1981).