Increases in Na⁺,K⁺-ATPase activity of erythrocytes and skeletal muscle after chronic ethanol consumption: Evidence for reduced efficiency of the enzyme

(alcoholism/ouabain binding/glycolysis)

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ABSTRACT Increased Na⁺,K⁺-ATPase activity observed after chronic ethanol consumption has been examined to determine whether the increase is due to changes in the kinetic properties of the enzyme or increases in the amount of enzyme in the membranes examined. In skeletal muscle and erythrocyte ghosts from rat, as well as from humans, increased Na⁺,K⁺-ATPase activity in ethanol-consuming individuals was not accompanied by an increase in the number of ouabain binding sites. In studies with intact human erythrocytes, similar ouabain-sensitive 22Na⁺ and 86Rb⁺ pumping rates were observed between normal and ethanol-consuming individuals and the Na⁺ to Rb⁺ pumping ratio was found to be 1.5 in all cases. However, ouabain-sensitive lactate plus Pi formation was increased in cells from alcoholic individuals. Thus these data suggest that increased enzyme activity may be due to a kinetic alteration of the Na⁺,K⁺-ATPase and that the enzyme may be less efficient in coupling ion pumping to ATP hydrolysis than the enzyme in normal cells.

Experimental models examining the effects of alcoholism on cation transport have resulted in persistent reports that Na⁺,K⁺-ATPase activity of skeletal muscle (1), erythrocyte (2, 3), brain (2, 4), and liver (5) plasma membranes is increased. This increase has been postulated to be a secondary consequence of decreased membrane resistance and increased membrane sodium permeability that result in a rise in intracellular sodium concentrations (1, 6, 7). Because these measurements have been made under conditions assessing V_max of the Na⁺,K⁺-ATPase, the simplest explanation for increased activity is an increase in the number Na⁺,K⁺-ATPase pumps in the plasma membrane (1–7).

In testing this hypothesis, we have measured Na⁺,K⁺-ATPase activity and ouabain binding in rat skeletal muscle and in intact erythrocyte and ghost preparations from rats and humans after chronic ethanol consumption. Although all criteria used for evaluating ATP hydrolysis rates indicate increases in Na⁺,K⁺-ATPase activity, which is quantitatively similar to previous findings (1–3) from alcoholic individuals, the ion pumping rates, the ion pumping ratios, and the number of ouabain binding sites were not different from controls. These data suggest a kinetic alteration of the enzyme consistent with less efficient coupling of ATP hydrolysis to ion pumping.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 200–300 g (Sasco, Omaha, NE) were fed 7% (vol/vol) ethanol ad libitum as a liquid part of their diet for 28 days. Age-matched control animals of equivalent size were fed the same diet supplemented with Polycose (Ross Laboratories, Columbus, OH) to approximate caloric differences in the two test groups. Animals were anesthetized by injection with 100 mg of inactin per kg (body weight) and 10 ml of blood was collected in syringes containing 1 ml of acid citrate/dextrose as an anticoagulant. Hind-limb skeletal muscle [5 g (wt weight)] was collected and visible fat and connective tissue were removed by sharp dissection. We have described (8) the skeletal muscle microsomal preparation.

Human volunteers were selected from those admitted to the Veterans Administration Medical Center Alcohol Treatment Program who were rated "severe" for alcoholism according to research diagnostic criteria (9). Other criteria for selection included the absence of liver disease and no history of cardiac glycoside therapy. Normal individuals were selected using similar criteria except that alcohol consumption was less than 20 g/day. The mean age of alcoholic individuals was 38 years and the mean age of normal individuals was 34 years.

Erythrocytes were washed three times in 0.9% NaCl with a 5-min centrifugation at 600 × g after each wash. The buffy coat of leukocytes was carefully removed after each wash. Erythrocytes were then suspended in 50 mM Hepes (pH 7.4) containing 10 mM glucose, 105 mM NaCl, 5 mM KCl, 4 mM sodium phosphate, 5 mM adenosine, and 1 mM MgCl₂. The suspension was divided into two portions and one portion received sufficient 22NaCl to give a final specific activity of 0.8 Bq/nmol of Na⁺. After an overnight incubation at 4°C with shaking, both portions were washed in unlabeled buffer without glucose, phosphate, or adenosine and collected by centrifugation at 800 × g for 10 min at 4°C. A 200-μl aliquot of the unlabeled cells was removed, washed with 150 mM choline chloride, and retained for intracellular Na⁺ determinations using flame photometry (8). A second aliquot (approximately 10 ml of packed erythrocytes) was obtained for ghost preparation and ouabain binding determinations.

Assays of ouabain-sensitive 22Na efflux, ouabain-sensitive 86Rb uptake, and ouabain-sensitive lactate plus Pi formation are similar to the procedures used in the classical experiments of Whitlam and Ager (10), except that ion fluxes were measured with the use of isotopes after passage of cells through a Tris/Dowex column (11). Details of the analysis of lactate including a slight modification in the neutralization procedure are published elsewhere (12). Rates of ouabain-sensitive ion pumping and lactate plus Pi formation were determined at time zero and 10 min, 20 min, and 30 min after

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the addition of glucose (final concentration, 10 mM) or, where appropriate, glucose plus $^{86}$Rb (1.6 Bq/nmol of K$^+$.)

Unsealed erythrocyte ghosts were prepared by the procedures of Steck and Kant (13). Assays of ouabain-sensitive ATP hydrolysis by erythrocyte ghosts and skeletal muscle microsomes utilized the procedures of Jorgenson (14).

Assays and analysis of magnesium-vanadate-facilitated $[^3]$Houabain binding have been described by this laboratory (8) except that ouabain binding to erythrocytes was determined using eight concentrations of ouabain from 0.50 to 100 $\mu$M. Also, intact erythrocytes were lysed with 10% (wt/vol) SDS, heated at 90°C for 10 min, and decolorized with 100 $\mu$L of H$_2$O$_2$ prior to analysis of $[^3]$Houabain binding.

ATPase assays were performed in a medium containing 120 mM NaCl, 5 mM KCl, 2 mM MgCl$_2$, and 50 mM Tris-HCl (pH 7.4). After a 5-min equilibration at 37°C, vanadum-free Tris/ATP was added to a final concentration of 3 mM and the incubation was continued for 20 min. The reaction was terminated by the addition of trichloroacetic acid. P$_1$ and protein were determined as described (8). Na$^+$,K$^+$-ATPase was defined as the difference between P$_1$ liberated under the above condition and P$_1$ liberated in matched samples assayed in the absence of NaCl and KCl in the presence of 1 mM ouabain (8).

Statistical comparisons between test groups used the unpaired Student's t test, and significance was defined by $P < 0.05$.

**RESULTS**

Table 1 shows that after 28 days of ethanol consumption, skeletal muscle microsomal Na$^+$,K$^+$-ATPase activity from ethanol-fed rats was 5.2 $\pm$ 0.7 $\mu$mol of P$_1$ per mg of protein per hr as compared to 2.4 $\pm$ 0.4 $\mu$mol of P$_1$ per mg of protein per hr for microsomes from Polyco-supplemented rats. This activity increase is qualitatively and quantitatively similar to the increase reported for the alcoholic dog (1). Using data for $B_{max}$ of ouabain binding, increased Na$^+$,K$^+$-ATPase density did not account for the activity increase observed and we concluded that skeletal muscle microsomes from ethanol-fed rats hydrolyzed more ATP per ouabain binding site than microsomes from normal individuals (Table 1).

An analogous result was obtained with erythrocyte ghosts from these animals. In the ethanol-fed rat, Na$^+$,K$^+$-ATPase activity was 4.0 $\pm$ 0.7 $\mu$mol of P$_1$ per mg of protein per hr compared to 2.2 $\mu$mol of P$_1$ per mg of protein per hr from Polyco-supplemented rats (Table 1). Activity of the Na$^+$,K$^+$-ATPase per ouabain binding site was observed to be increased.

To further explore the discrepancy between ouabain binding and ouabain-sensitive ATP hydrolysis, the experimental approach of Whittam and Ager (10) was employed to estimate the ouabain-sensitive ion transport rates and ATP hydrolysis rates of human erythrocytes. These authors took advantage of the high glycolytic rates of erythrocytes to obtain a measurement of ATP hydrolysis by observing ouabain-sensitive lactate plus P$_1$ formation. Using this approach, these authors were able to confirm the 3:2:1 ratio for Na$^+$ efflux, K$^+$ uptake, and ATP hydrolysis by the Na$^+$,K$^+$-ATPase in human erythrocytes (10).

Data in Table 2 show that approximately 80% of the sum of ouabain-sensitive lactate plus P$_1$ rates in erythrocytes from normal individuals is lactate. Further, the data for ouabain-sensitive lactate plus P$_1$ rates in normal erythrocytes are virtually identical to those reported by Whittam and Ager (10).

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Microsomes</th>
<th>Ghosts</th>
<th>Microsomes</th>
<th>Ghosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.4 ± 0.4</td>
<td>2.2 ± 0.6</td>
<td>1.4 ± 0.3</td>
<td>0.49 ± 0.10</td>
</tr>
<tr>
<td>Alcoholic</td>
<td>5.2 ± 0.7*</td>
<td>4.0 ± 0.7*</td>
<td>1.5 ± 0.3</td>
<td>0.51 ± 0.11</td>
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</table>

Preparation of membranes and conditions for determining Na$^+$,K$^+$-ATPase activity and $[^3]$Houabain binding are given in Materials and Methods and ref. 8. Values reported are mean ± SEM ($n$ = 12 for all samples). *$P < 0.05$.

Table 1. Na$^+$,K$^+$-ATPase activity and ouabain binding in skeletal muscle membranes and erythrocyte ghosts from control and ethanol-fed rats

<table>
<thead>
<tr>
<th>Sample</th>
<th>Condition</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Intact erythrocyte</td>
<td>Lactate formation, $\mu$mol per ml per hr</td>
</tr>
<tr>
<td></td>
<td>P$_1$ formation, $\mu$mol per ml per hr</td>
</tr>
<tr>
<td></td>
<td>$^{22}$Na efflux, $\mu$mol per ml per hr</td>
</tr>
<tr>
<td></td>
<td>$^{86}$Rb uptake, $\mu$mol per ml per hr</td>
</tr>
<tr>
<td></td>
<td>Ratio of $^{22}$Na efflux/$^{86}$Rb uptake</td>
</tr>
<tr>
<td></td>
<td>Ratio of $^{22}$Na efflux/formation of lactate plus P$_1$</td>
</tr>
<tr>
<td></td>
<td>Ratio of $^{86}$Rb uptake/formation of lactate plus P$_1$</td>
</tr>
<tr>
<td></td>
<td>OBS, $\mu$mol/ml of erythrocytes</td>
</tr>
<tr>
<td></td>
<td>Ratio of $^{22}$Na efflux/OBS, mol of $^{22}$Na per min per mol of ouabain bound</td>
</tr>
<tr>
<td></td>
<td>Ratio of $^{86}$Rb uptake/OBS, mol of $^{86}$Rb per min per mol of ouabain bound</td>
</tr>
<tr>
<td></td>
<td>Ratio of formation of lactate plus P$_1$/OBS, mol of lactate plus P$_1$ per min of mol of ouabain bound</td>
</tr>
</tbody>
</table>

Erythrocyte ghosts

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal</th>
<th>Alcoholic</th>
</tr>
</thead>
<tbody>
<tr>
<td>OBS, $\mu$mol of protein</td>
<td>0.35</td>
<td>0.39</td>
</tr>
<tr>
<td>ATPase activity, $\mu$mol per hr per mg of protein</td>
<td>0.11 (0.05)</td>
<td>0.22 (0.09)</td>
</tr>
<tr>
<td>Ratio of ATPase/OBS, mol of P$_1$ per min per mol of ouabain bound</td>
<td>5238</td>
<td>9401</td>
</tr>
</tbody>
</table>

Table 2. Na$^+$,K$^+$-ATPase activity of erythrocytes from normal and alcoholic humans

$^{86}$Rb uptake and $^{22}$Na efflux, ouabain-sensitive formation of lactate and P$_1$, and ouabain binding sites (OBS) were measured in intact human erythrocytes. Ouabain binding sites and Na$^+$,K$^+$-ATPase activity were measured in human erythrocyte ghosts. See Table 1 for more details. Data are the mean of duplicate determinations from six individuals; numbers in parentheses are SEM.

* $P < 0.05$.

$^t0.05 < P < 0.2$.
under similar experimental conditions. When these data are paired with \(^{22}\text{Na}\) efflux data measured simultaneously in preloaded erythrocytes, the ratio of \(^{22}\text{Na}\) efflux to lactate plus \(P_i\) is 3.10 in erythrocytes from normal individuals (Table 2). A second fraction of these same erythrocytes revealed that the ratio of ouabain-sensitive \(^{86}\text{Rb}\) uptake to ouabain-sensitive lactate plus \(P_i\) formation was 2.05 (Table 2). These data are in excellent agreement with the 3:2:1 ratio of \(\text{Na}^+\) efflux, \(\text{K}^+\) uptake, and ATP hydrolysis of the \(\text{Na}^+,\text{K}^+\)-ATPase (10, 15).

Also shown in Table 2 are determinations of ouabain-sensitive \(^{22}\text{Na}\) efflux, \(^{86}\text{Rb}\) uptake, and lactate plus \(P_i\) formation rates by using erythrocytes from alcoholic humans. Each experiment was performed side-by-side with erythrocytes from normal humans under identical conditions. Erythrocytes from alcoholic humans display a significant increase in ouabain-sensitive lactate formation rates that result in an increase in the ouabain-sensitive lactate plus \(P_i\) component of the analysis. However, ouabain-sensitive \(^{86}\text{Rb}\) uptake and \(^{22}\text{Na}\) efflux are indistinguishable from rates measured in erythrocytes from normal humans (Table 2). This results in a decrease in the calculated ratios of \(\text{Na}^+\) or \(\text{Rb}^+\) to ouabain-sensitive lactate plus \(P_i\) formation from 3.10 to 2.09 and from 2.05 to 1:43, respectively.

When these data are combined with ouabain-binding data for intact erythrocytes to reflect rates of \(\text{Na}^+\text{flx}, \text{K}^+\text{uptake, and lactate plus } P_i\text{ formation per ouabain binding site, the following conclusions can be drawn. (i) The numbers of } \text{Na}^+\text{ and } \text{Rb}^+\text{ pumped per ouabain binding site were lower in erythrocytes from alcoholic individuals than in erythrocytes from normal individuals. (ii) Lactate plus } P_i\text{ formation rates per ouabain binding site were higher in erythrocytes from alcoholic individuals than from normal individuals. (iii) The ratios of } \text{Na}^+\text{ efflux to } \text{Rb}^+\text{ uptake were not altered from the 1.5 ratio observed in erythrocytes from normal humans, indicating that the electrogenic nature of the enzyme was preserved. (iv) When membrane ghosts were prepared from these cells, ATP hydrolysis rates from alcoholic individuals were observed to be higher while the number of ouabain binding sites was not increased (Table 2).}

These data can be interpreted to mean that erythrocytes from alcoholic individuals hydrolyze more ATP to maintain similar ion pumping rates and the 1.5 \(\text{Na}/\text{Rb}\) ratio observed in normal erythrocytes. Because the number of ouabain binding sites in these membranes cannot account for observed activity increases, we conclude that a portion of the \(\text{Na}^+,\text{K}^+\)-ATPase activity in erythrocytes from alcoholic individuals may not tightly couple ion pumping and ATP hydrolysis.

Even though the indirect assay of ATP hydrolysis in intact cells is corroborated by measurements of ATP hydrolysis in ghost preparations, an alternate explanation for these data is that intact erythrocytes from alcoholic individuals may mobilize other triose phosphates, particularly 2,3-bisphosphoglycerate, for glycolysis. No evidence for increased levels of 2,3-bisphosphoglycerate or turnover of 2,3-bisphosphoglycerate were found in these experiments (data not shown). Secondly, no increased leakage of \(^{22}\text{Na}^+\) (as judged by ouabain-insensitive efflux) or \(^{86}\text{Rb}\) could be demonstrated in these cells (Fig. 1).

**DISCUSSION**

Our data are consistent with numerous reports of increased \(\text{Na}^+,\text{K}^+\)-ATPase activity in several tissues after chronic alcohol consumption (1–5). In skeletal muscle, a temporal relationship has been shown between activity alterations and increased sodium permeability (1). The suggestion has been made that kinetic alterations are secondary to increased intracellular sodium concentrations and are best explained by increased numbers of \(\text{Na}^+,\text{K}^+\)-ATPase pumps in the membrane (1, 6, 7). The question of whether increases in \(\text{Na}^+,\text{K}^+\)-ATPase activity are the result of altered kinetic properties of the enzyme or increased numbers of pumps has remained open except for one report of anatomically discrete increases in ouabain binding to brain tissue from cats (16).

A second possibility is that these activity alterations are due to improper processing of the pump, insertion of the pump into the membrane, or synthesis of different isoforms of the pump, since the time course of activity changes are consistent with the synthesis of new protein in skeletal muscle (1). The latter possibility is intriguing since the

![Graph](image-url)

**Fig. 1.** Ouabain-insensitive \(^{22}\text{Na}\) efflux (A) and \(^{86}\text{Rb}\) leakage (B) from erythrocytes of normal (○) and alcoholic (●) individuals. (A) Erythrocytes (RBCs) were loaded with \(^{22}\text{Na}\) overnight and ouabain-insensitive \(^{22}\text{Na}\) efflux was defined as the time-dependent release of \(^{22}\text{Na}\) in cells treated with 1 mM ouabain. Intracellular \(\text{Na}^+\) levels were determined and were 6.2 mM in cells from normal individuals and 6.9 mM in cells from alcoholic individuals. (B) Duplicate samples of erythrocytes from alcoholic individuals were loaded for 1 hr at 37°C with \(^{86}\text{Rb}\), washed twice, and incubated in buffers used for glycolysis at 37°C. At the times indicated, aliquots were passed through Tris/Dowex columns. At 60 min, 4 \(\mu\text{M}\) nigericin was added to one sample to demonstrate release of \(^{86}\text{Rb}\) by these cells.
isofoms of the pump have been thought to predominate in neurons and muscle cells until a recent report (17) showing A3 isofom expression in human hematopoietic cells. These authors also present evidence that the modulation of A3 mRNA levels may be posttranscriptional. Given the tissue distribution of isofoms of the enzyme and the fact that activity differences, if any exist, have not been characterized for these isofoms, perhaps isofom expression differences could account for the alterations of Na⁺,K⁺-ATPase activity observed.

It is also possible that these changes in activity are in response to disruption of critical lipid–protein interactions that are the result of known effects of ethanol on membrane lipid composition and fluidity (18). Swann and coworkers (3, 19) have presented evidence that alterations in Na⁺,K⁺-ATPase activity may represent a direct kinetic adaptation to the effects of ethanol on membrane fluidity.

Data presented here support the hypothesis that increased activity of the enzyme in skeletal muscle microsomes and erythrocytes may be the result of altered kinetic properties of the enzyme rather than increased amounts of enzyme. The evidence for this is as follows: (i) ATP hydrolysis measured under \(V_{\text{max}}\) conditions with our membrane preparations is increased without a concomitant increase in the number of ouabain binding sites. (ii) Rates of intact human erythrocyte ouabain-sensitive \(^{22}\text{Na}^+\) and \(^{86}\text{Rb}^+\) pumping are similar between normal and alcoholic individuals, but ouabain-sensitive lactate plus P₄ formation rates are increased in erythrocytes from alcoholic individuals. The number of ouabain binding sites in intact erythrocytes from alcoholic individuals is not significantly different from controls. (iii) No evidence for \(^{22}\text{Na}^+\) or \(^{86}\text{Rb}^+\) leakage from intact cells could be demonstrated that could account for these differences.

It should be noted that the \(^{22}\text{Na}^+\) to \(^{86}\text{Rb}^+\) pumping ratio in erythrocytes from alcoholic individuals is close to the established value of 1.5 for the Na⁺,K⁺-ATPase and observed in cells from normal individuals. Because the ion pumping ratios and rates of erythrocytes from both groups are similar, it is conceivable that erythrocytes from ethanol-consuming individuals maintain normal Na⁺,K⁺-ATPase-dependent ion gradients but do so at the expense of ATP.

Although it has not been possible to make similar measurements in skeletal muscle, all measurements common to erythrocytes and skeletal muscle Na⁺,K⁺-ATPase suggest that analogous alterations in skeletal muscle enzyme occur. If this is true, these results may explain the precipitation of clinical alcoholic myopathy during periods of heavy metabolic stress by disruption of normal ion gradients in muscle because of the added ATP costs of maintaining them (1, 7, 20).