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REGULATION OF Na^+,K^+-ATPase BY CHRONIC ETHANOL EXPOSURE OF PC 12 CELLS

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Abstract—The effects of chronic ethanol exposure on Na^+,K^+-ATPase were investigated in PC 12 cells. Inclusion of ethanol in the Na^+,K^+-ATPase assay (i.e. *in vitro* addition of ethanol) inhibited enzyme activity. Conversely, intrinsic Na^+,K^+-ATPase activity was increased after chronic ethanol exposure of the cells. This increase in Na^+,K^+ pumps occurred without any alteration in the inhibitory effects of *in vitro* ethanol. A similar response was observed when the chronic treatments were carried out using serum-free defined medium. The effects of other agents, which like ethanol decrease membrane order, were investigated. The addition of ketamine and *tert*-butanol *in vitro* caused a concentration-dependent inhibition of Na^+,K^+-ATPase activity. However, chronic exposure of the PC 12 cells to *tert*-butanol or ketamine did not alter either intrinsic Na^+,K^+-ATPase activity or the inhibitory effects of ethanol *in vitro*. Maintenance of PC 12 cells in medium containing ethanol resulted in an increase in the intracellular content of Na^+ without any change in the K^+ levels. In contrast, maintenance of the cells in medium containing *tert*-butanol did not alter intracellular levels of Na^+ or K^+. The present study shows that the ethanol-induced increase in Na^+,K^+ pumps involved an increase in the intracellular content of Na^+. This increase in Na^+ content did not appear to be secondary to an inhibition of Na^+,K^+-ATPase activity.

Na^+,K^+-ATPase is a transmembrane protein that maintains cellular ionic gradients, osmotic balance and membrane electrical potential by the counter-transport of Na^+ and K^+. The activity of this Na^+,K^+ pump is altered by hormones and drugs including ethanol. In a variety of cell and tissue preparations the *in vitro* addition of ethanol (i.e. inclusion of alcohol in the assay) causes an inhibition of Na^+,K^+-ATPase activity [1–10]. This inhibitory effect of *in vitro* ethanol has been reported to occur with alcohol concentrations as low as 100 mg/dL [10] and is antagonized in a competitive-like fashion by KCl [2, 3, 7, 11]. In cultures of rat myotubes, ethanol-induced membrane depolarization is secondary to the inhibition of Na^+,K^+-ATPase activity [9].

Prolonged exposure to ethanol has been reported to have two effects on the Na^+,K^+ pump. Chronic treatment of rodents with ethanol has been shown to reduce the inhibitory effect of *in vitro* ethanol on Na^+,K^+-ATPase activity [4, 5, 12]. In addition, chronic administration of ethanol also was reported to increase Na^+,K^+-ATPase activity in the brain [11–14], but this has not been a consistent finding [4, 5, 15]. The effects of chronic ethanol exposure on Na^+,K^+-ATPase, however, appear to vary among the different brain regions [16, 17]. Furthermore, the effects of chronic ethanol exposure also may be cell specific. Thus, an increase in Na^+,K^+-ATPase activity was observed in PC 12 cells [11], rat skeletal myotubes [18], chick glial cells [19] and in N1E-115 neuroblastoma cells [20] after chronic exposure to ethanol, but not in MDCK cells [11] or in either rat glial cells or the NN line of astroblasts [20].

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In PC 12 cells, which have a number of neuronal characteristics, the increase in Na^+,K^+-ATPase activity is due to an increase in the cellular density of Na^+,K^+ pumps [11]. An up-regulation of the Na^+,K^+ pump has also been observed with prolonged exposure of cells to ouabain and low extracellular potassium [21–27]. In both these cases the increase in the cellular density of Na^+,K^+-ATPase was suggested to be secondary to an increase in the intracellular concentration of Na^+ [21–25]. The present study was undertaken to investigate how chronic ethanol exposure of PC 12 cells alters Na^+,K^+-ATPase activity.

MATERIALS AND METHODS

Fetal bovine serum was obtained from Sterile Systems Inc. (Logan, UT) and heat-inactivated horse serum was obtained from JRH Bioscience (Lenexa, KS). RPMI 1640 was obtained from Gibco Laboratories (Grand Island, NY). All other materials were purchased through common commercial sources.

Tissue culture. PC 12 cells were maintained at 37° on 100 mm plastic tissue culture plates in 85% RPMI containing 5% fetal bovine serum and 10% heat-inactivated horse serum in an atmosphere of 95% air–5% CO_2. Incubation of PC 12 cells in serum-free defined medium was carried out using the N2 growth medium developed by Bottenstein and Sato [28] except that Dulbecco's Modified Eagle's Medium (DMEM) was replaced with RPMI 1640 and 10 mM Hepes (pH 7.4) was included (i.e. RPMI/N2). Because PC 12 cells were found to require serum for attachment to the tissue culture plates, cells were subcultured in the presence of serum and switched to the RPMI/N2 medium after 24 hr. RPMI/N2 containing ethanol was added after an additional

24 hr (i.e. 48 hr after subculture). For these experiments the plastic tissue culture dishes were coated with ammoniated collagen. When PC 12 cells were exposed to ethanol, ketamine or *tert*-butanol, these drugs were added 24 hr after the cells were subcultured. In each set of experiments sister cultures were randomly divided into the appropriate treatment and control groups. All treatments with ketamine or the alcohols were carried out for a total of 4 days at 37° with medium replaced after 2 days. Chronic treatment of cells with the alcohols was carried out using plastic desiccators containing a 95% air–5% CO_2 atmosphere that was saturated with the appropriate concentration of alcohol to minimize evaporation [11]. Over a 4-day period the loss of ethanol was 14.2 ± 2.6% (N = 13) and the loss of *tert*-butanol was 26.8 ± 6.6% (N = 8).

Na^+,K^+-*ATPase assay*. After the 4-day drug treatment, PC 12 cells were harvested in phosphate-buffered saline by trituration and were rinsed twice in phosphate-buffered saline by centrifugation at 800 *g* for 4 min. The pellet of washed PC 12 cells was incubated in 10 mM Tris–HCl (pH 7.5) at 4° for 5 min and then was homogenized with a Dounce tissue grinder (Wheaton pestle B). An equal volume of 10 mM Tris–HCl (pH 7.5) containing 20% (w/v) sucrose was added, and the homogenate was centrifuged at 5,000 *g* for 20 min at 4°. No difference in the recovery of Na^+,K^+-ATPase activity was observed with centrifugation at either 5,000 *g* or 20,000 *g* [11]. The pellet was resuspended in 10 mM Tris–HCl (pH 7.5) containing 10% sucrose and was again centrifuged at 5,000 *g* for 20 min. The resulting pellet was resuspended in the Tris/sucrose buffer and was preincubated for 30 min at room temperature with an equal volume of 48 mM imidazole buffer (pH 7.0) containing 3.8 mM EDTA and 0.028% (w/v) deoxycholate.

Na^+,K^+-ATPase activity was determined by measuring the release of inorganic phosphorus from ATP. The ATPase assay was carried out at 37° in a final volume of 800 μL consisting of 100 μL tissue (3–11 μg DNA) and 30 mM histidine buffer (pH 7.5) containing 3 mM vanadate-free ATP, 3 mM $MgCl_2$, and either 130 mM NaCl and 20 mM KCl or 1 mM ouabain. Reactions were initiated by the addition of prewarmed $MgCl_2$/ATP and were terminated after 20 min with 100 μL of cold 8.3% (w/v) perchloroacetic acid. The amount of inorganic phosphorus released was determined by the method of Fiske and Subbarow [29]. Na^+,K^+-ATPase activity represents the difference between activity measured in the absence and presence of ouabain.

Intracellular ion content. Intracellular sodium and potassium ion concentrations were measured as described by Pressley *et al.* [26]. Briefly, cells were rinsed rapidly five times in ice-cold 150 mM ammonium acetate, and the cells were removed in 3 mM $LiNO_3$ by scraping with a rubber policeman. The plates were rinsed with additional $LiNO_3$, and the samples were frozen at −20°. The samples were thawed and cellular sodium and potassium ion content determined by flame photometry after centrifugation at 10,000 *g* for 15 min to remove any particulates. Initial studies indicated that all cells were lysed by the scraping and freeze–thawing.

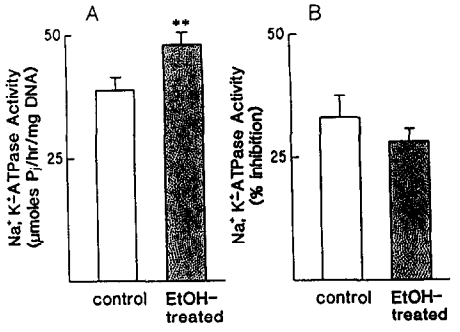

Fig. 1. Effects of chronic ethanol exposure of PC 12 cells on Na^+,K^+-ATPase activity. PC 12 cells were treated for 4 days with 150 mM ethanol as described in Materials and Methods. (A) Intrinsic Na^+,K^+-ATPase activity measured in the absence of ethanol *in vitro*. (B) Per cent inhibition of Na^+,K^+-ATPase activity by the addition of *in vitro* ethanol (500 mM). Data are plotted as the means ± SEM of 7 separate experiments. Key: (**) $P < 0.01$ (paired *t*-test).

DNA content was determined by the spectrofluorometric method of Labarca and Paigen [30].

RESULTS

Treatment of PC 12 cells with 150 mM ethanol for 4 days significantly increased Na^+,K^+-ATPase activity (Fig. 1). However, the inhibitory effect of ethanol *in vitro* on enzyme activity was not altered by the chronic treatment. Because of the competitive-like interaction of KCl and ethanol on Na^+,K^+-ATPase activity [2, 3, 7, 11] and the use of 20 mM KCl in the present assays, high *in vitro* concentrations of ethanol were required to observe an inhibition of Na^+,K^+-ATPase activity. When a more physiological concentration of KCl (5 mM) is used in the assay, Na^+,K^+-ATPase activity is inhibited at lower concentrations of *in vitro* ethanol [11]. However, in the present study 20 mM KCl was used to facilitate determining intrinsic Na^+,K^+-ATPase activity because chronic ethanol exposure increased the cellular density of Na^+,K^+-ATPase without altering the inhibitory action of ethanol *in vitro* on the enzyme.

Serum contains a number of factors that regulate Na^+,K^+-ATPase activity [31, 32]. The potential role of serum factors in the observed ethanol-induced increase in Na^+,K^+-ATPase was investigated by treating cells with ethanol using serum-free defined medium. Exposure of PC 12 cells to 100 mM ethanol for 4 days using serum-free defined medium resulted in a significant increase in Na^+,K^+-ATPase activity (Fig. 2). The inhibitory effects of *in vitro* ethanol on enzyme activity, however, were not altered by the chronic treatment.

The increase in Na^+,K^+-ATPase activity after chronic ethanol exposure could be compensation for the acute inhibitory effects of ethanol. This possibility was investigated by chronically exposing PC 12 cells to *tert*-butanol and ketamine. Both these compounds, like ethanol, cause a decrease in membrane order

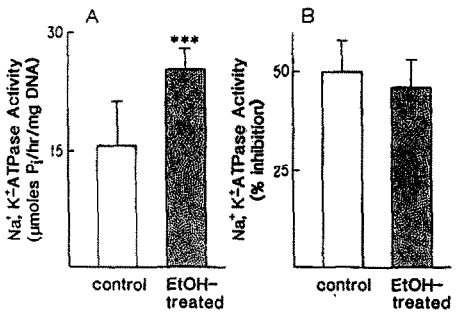

Fig. 2. Effects of chronic ethanol exposure using serum-free defined medium on Na^+,K^+-ATPase activity. PC 12 cells were treated for 4 days with 100 mM ethanol using the RPMI/N2 medium as described in Materials and Methods. (A) Intrinsic Na^+,K^+-ATPase activity measured in the absence of ethanol *in vitro*. (B) Per cent inhibition of enzyme activity by the addition of ethanol (500 mM) *in vitro*. Data are plotted as the means ± SEM of 8 separate experiments. Key: (***) $P < 0.001$ (paired *t*-test).

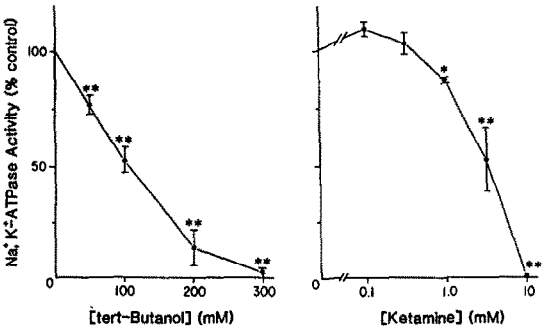

Fig. 3. Effects of *in vitro tert*-butanol and ketamine on Na^+,K^+-ATPase activity. Data are plotted as means ± SEM of 4 separate experiments. Na^+,K^+-ATPase activity in the absence of drugs was 21.1 ± 2.5 μmol P_i/hr/mg DNA. Key: (*) $P < 0.05$, and (**) $P < 0.01$ (Dunnett's test for multiple comparisons) compared to activity in the absence of drug.

[33–35]. Furthermore, the *in vitro* addition of ketamine and *tert*-butanol also caused a concentration-dependent decrease in Na^+,K^+-ATPase activity (Fig. 3). A 4-day exposure to 50 mM *tert*-butanol or 1 mM ketamine, however, did not alter Na^+,K^+-ATPase activity (Table 1; $F_{2,17} = 1.459$). Similarly, chronic exposure to these compounds did not alter the inhibitory effects of either 250 mM ($F_{2,17} = 0.430$) or 500 mM ethanol *in vitro* ($F_{2,18} = 3.178$).

The increase in Na^+,K^+-ATPase activity after incubation of cells in ouabain or in medium containing low extracellular potassium has been suggested to be due to an increase in intracellular sodium ion content [21–25]. The effects of chronic ethanol exposure on intracellular sodium and potassium ion contents in PC 12 cells were therefore investigated. An 8-hr exposure to 150 mM ethanol had no statistically significant effect on intracellular sodium content (9.02 ± 0.45 and 9.38 ± 1.64 nmol/μg DNA for control and ethanol-treated cells, respectively; N = 3) or on intracellular potassium content (24.6 ± 1.4 and 22.6 ± 0.48 nmol/μg DNA for control and ethanol-treated cells, respectively; N = 3). Longer exposure to ethanol resulted in a significant increase in sodium content (Fig. 4). Two-way ANOVA indicated a highly significant increase in sodium content in the ethanol-treated cells ($P = 0.008$). There was no effect of time ($P = 0.193$) and there was no interaction between treatment and time ($P = 0.501$). The ethanol treatment did not alter intracellular potassium content ($P = 0.453$) and there was neither an effect of time ($P = 0.249$) nor an interaction between treatment and time ($P = 0.87$). A 4-day exposure to 50 mM *tert*-butanol did not alter intracellular sodium content ($P = 0.422$) and there was neither an effect of time ($P = 0.791$), nor an interaction between treatment and time ($P = 0.682$) (Fig. 5). Similarly, *tert*-butanol exposure did not alter intracellular potassium content ($P = 0.951$) and there was neither an effect of time ($P = 0.545$) nor an interaction between treatment and time ($P = 0.974$).

DISCUSSION

The number of Na^+,K^+ pumps per cell is increased by a variety of conditions including prolonged incubation with ouabain, veratridine, low extracellular potassium and hormones such as aldosterone and thyroid hormone [21–25, 36–39]. An increase in Na^+,K^+-ATPase activity also occurs after chronic exposure of PC 12 cells to ethanol. This increase in enzyme activity was shown previously to be due to an increase in the number of Na^+,K^+ pumps per cell

Table 1. Effects of chronic exposure to *tert*-butanol and ketamine on Na^+,K^+-ATPase activity

	Na^+,K^+-ATPase activity		
	(μmol/P_i/hr/mg DNA)	% Inhibition by 250 mM EtOH	% Inhibition by 500 mM EtOH
Control	40.0 ± 4.1	10.1 ± 1.2	27.1 ± 2.2
t-Butanol	52.2 ± 4.6	12.8 ± 2.0	39.1 ± 3.9
Ketamine	43.5 ± 7.4	12.8 ± 4.0	32.2 ± 4.0

PC 12 cells were exposed to 50 mM *tert*-butanol or 1 mM ketamime for 4 days as described in Materials and Methods. Data are expressed as means ± SEM of 6–8 separate experiments.

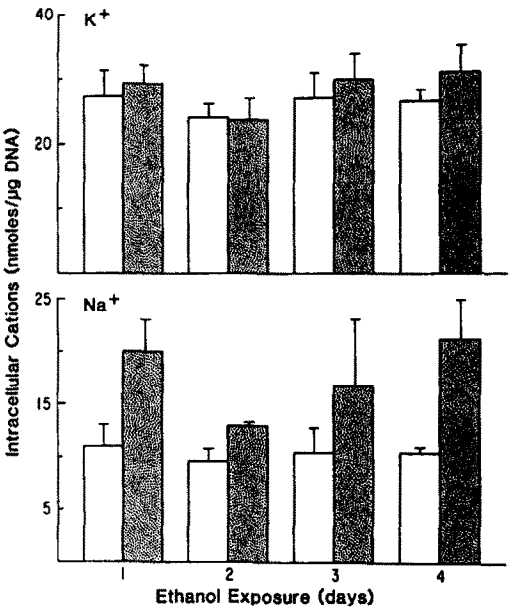

Fig. 4. Time course for ethanol-induced changes in intracellular sodium and potassium ion content. Intracellular potassium (top) and sodium (bottom) ion contents were determined in PC 12 cells incubated in the absence (open bars) or presence (stippled bars) of 150 mM ethanol for various lengths of time. Data are plotted as the means ± SEM of 4–6 separate experiments. Two-way ANOVA was carried out using the SPSS/PC program.

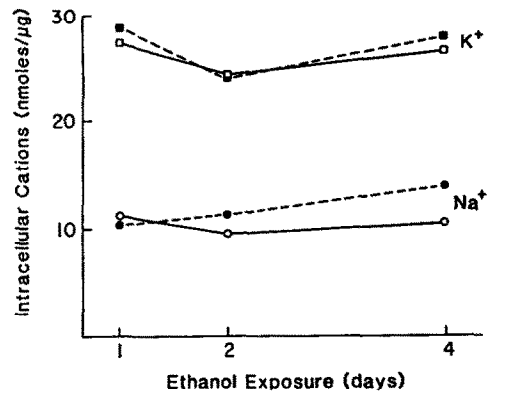

Fig. 5. Effects of *tert*-butanol exposure on intracellular sodium and potassium ion content. Intracellular potassium and sodium contents were determined in PC 12 cells incubated in the absence (open symbols) or presence (closed symbols) of 50 mM *tert*-butanol for various lengths of time. Data are plotted as the means ± SEM of 3–6 separate experiments; SEM were less than 35% of the mean. Two-way ANOVA was carried out using the SPSS/PC program.

[11]. Although serum has been shown to alter Na^+,K^+-ATPase [31, 32], the observed increase does not require the growth factors in the serum since an increase in Na^+,K^+-ATPase activity was observed with serum-free defined medium. A similar increase in Na^+,K^+-ATPase after chronic ethanol administration was reported in brain [11–14], but appears to be a region-specific change [16, 17]. Furthermore, the ethanol-induced increase in the cellular density of Na^+,K^+ pumps also may be cell-specific because an increase in Na^+,K^+-ATPase activity was found in some clonal cells after chronic ethanol exposure [11, 18–20], but not in others [11, 20]. Interestingly, maximal Na^+,K^+-ATPase activity was found to be higher in erythrocytes from alcoholic patients compared to control subjects [40]. Furthermore, this elevated Na^+,K^+ pump activity was reduced after 3 months of abstinence [40].

Because an up-regulation of Na^+,K^+ pumps was observed with cells in culture, the increase in the number of Na^+,K^+ pumps appears to involve a direct action of ethanol on the cell. In contrast, the reported reduction in the inhibitory effects of *in vitro* alcohol on brain Na^+,K^+-ATPase activity after chronic ethanol administration to rodents [4, 5, 12] does not appear to be due to a direct action of ethanol. Rather, because chronic exposure of PC 12 cells to ethanol did not alter the subsequent ability of the alcohol to inhibit enzyme activity, the previously observed reduction in the inhibitory effect of *in vitro* ethanol was probably secondary to one of the various *in vivo* actions of ethanol.

Because the *in vitro* addition of ethanol inhibits Na^+,K^+-ATPase activity, the observed increase in pump density after prolonged ethanol treatment would appear to be consistent with the expected compensatory response. Inhibition of Na^+,K^+-ATPase activity *per se*, however, does not appear to be involved in the ethanol-induced up-regulation of the Na^+,K^+ pump. Chronic exposure of PC 12 cells to ketamine and *tert*-butanol did not change Na^+,K^+-ATPase activity although the *in vitro* addition of both these agents inhibited enzyme activity.

In cultured cells an increase in intracellular Na^+ concentration has been suggested to be the signal modulating Na^+,K^+-ATPase levels after exposure to ouabain or low extracellular potassium [21–25]. Chronic ethanol exposure of PC 12 cells was found to increase the intracellular concentration of Na^+. A similar increase in skeletal muscle sodium content was observed in dogs after chronic ethanol administration [41]. Thus, it appears that the increase in Na^+ content may be involved in the ethanol-induced increase in Na^+,K^+ pumps.

The mechanism for the ethanol-induced increase in intracellular Na^+ content is unclear. This increase was not due to the inhibition of Na^+,K^+-ATPase activity. Based on the data in Fig. 3, chronic exposure of PC 12 cells to *tert*-butanol should have resulted in a 21% inhibition of Na^+,K^+-ATPase activity while a 16% inhibition of the enzyme would be observed with chronic ethanol administration [11]. Yet, chronic ethanol exposure increased the intracellular content of Na^+ whereas chronic treatment with *tert*-butanol did not. For comparison, therapeutic doses of the cardiac glycoside, ouabain, which elicits an up-regulation of Na^+,K^+ pumps after chronic exposure, acutely inhibits erythrocyte Na^+,K^+-ATPase activity only 15–23% [42]. In addition, the lack of a change in intracellular K^+ content after ethanol exposure also is not consistent with an increase in Na^+ content secondary to an inhibition

of Na^+,K^+-ATPase activity. The ethanol-induced elevation in intracellular Na^+ content could be due to an increase in the number of sodium channels. Although Na^+ influx through the voltage-sensitive sodium channel is inhibited by ethanol *in vitro* [43, 44], chronic exposure of rat skeletal muscle cultures to ethanol was reported to increase the number of sodium channels as indicated by an increase in the density of [^{3}H]saxitoxin binding sites [45]. However, chronic administration of ethanol to rats does not alter the binding of [^{3}H]batrachotoxin to the sodium channel, and batrachotoxin-stimulated $^{22}Na^+$ uptake into synaptosomes is reduced by the chronic treatment [46]. Because chronic ethanol treatment was found to increase the intracellular levels of Ca^{2+} in PC 12 cells (Kim W-K and Rabin RA, unpublished data), it is also possible that the elevated levels of intracellular Na^+ could be secondary to an increase in Ca^{2+}–Na^+ exchange. Alternatively, Coca and Garay [47] reported that ethanol increases both Na^+ leakage into human erythrocytes and the activity of the Na^+–H^+ exchanger.

In summary, an elevation in the intracellular concentration of Na^+ appeared to be a primary effect of chronic ethanol exposure, while the ethanol-induced increase in the number of Na^+,K^+ pumps appeared to be a compensatory response to this increase in Na^+. Further studies are needed to determine the mechanism by which chronic ethanol exposure elevates the intracellular concentration of Na^+.

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