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Brain Synaptosomal (Na^+ and K^+)ATPase Activity as an Index of Tolerance to Ethanol

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BEAUGÉ, F., H. STIBLER AND H. KALANT. *Brain synaptosomal (Na^+ and K^+)ATPase activity as an index of tolerance to ethanol.* PHARMACOL BIOCHEM BEHAV **18**: Suppl. 1, 519–524, 1983.—Variations of brain synaptosomal (Na^+ and K^+)ATPase activity and development of functional tolerance to ethanol were followed simultaneously throughout a chronic ethanol treatment. Inhibition of the enzyme activity by ethanol added *in vitro* in the presence of noradrenaline was also assayed. Adult male rats were rendered tolerant to ethanol by daily intragastric administration of doses of 3–6 g of ethanol/kg body wt. As assessed by the hypothermic effect and impairment of motor performance on a tilting plane after injection of a challenge dose of ethanol (3 g/kg body wt. IP), functional tolerance developed slowly, was demonstrable after 2 weeks of treatment and increased for up to 4 weeks. The two tolerance tests gave parallel and positively correlated results. Concurrently with the development of tolerance, the basal activity of brain synaptosomal (Na^+ and K^+)ATPase increased (7% to 18%) in preparations from ethanol treated animals when compared to those from starch-fed caloric controls, and there was less inhibition of the enzyme by ethanol added *in vitro* in the presence of noradrenaline. The time course of the appearance of these changes in enzyme activity were positively correlated with that of behavioral tolerance, strengthening a relationship between the phenomena events. The intensity of the noradrenaline-ethanol interaction with the membrane-bound (Na^+ and K^+)ATPase activity could be an index of the degree of tolerance to ethanol.

Ethanol Tolerance Synaptosomes (Na^+ + K^+)ATPase Noradrenaline Rat

(Na^+ + K^+)ATPase, a major membrane-bound enzyme in the brain and other organs, which is responsible for the maintenance of the cellular resting potential [8], has been studied extensively in attempts to elucidate the biochemical mechanisms responsible for tolerance to ethanol.

Tolerance may be demonstrable on the biochemical level in systems which are disrupted by the presence of ethanol. It has been shown repeatedly that (Na^+ + K^+)ATPase activity is inhibited by ethanol added *in vitro* [13, 18, 24] through a mechanism involving apparent competition with the ability of K^+ to stimulate activity. However, conflicting results have been obtained concerning the effect of chronic ethanol treatment on the brain (Na^+ + K^+)ATPase activity [11, 12, 13, 14] and the possible relationship between changes in enzyme activity and ethanol tolerance and dependence [1, 7, 29, 30, 35].

Recent reports by Levental and Tabakoff [23] and Rangaraj and Kalant [31] suggested that tolerance may be more closely linked to a reduction in the sensitivity of ATPase to inhibition by ethanol added *in vitro*, than to the level of ATPase activity in the absence of ethanol. Particularly relevant is the finding by Rangaraj and Kalant [17, 30, 31, 32] of an α-adrenoreceptor-mediated modulation of ethanol effects on rat brain (Na^+ + K^+)ATPase. Noradrenaline (NA) and other α-adrenergic agonists sensitize this enzyme to inhibition by low concentrations of ethanol that do not otherwise produce any inhibition. Furthermore, after chronic ethanol treatment, the NA-induced sensitization to ethanol is markedly reduced [31]. If this phenomenon is linked to tolerance development rather than to ethanol withdrawal, the reduction of sensitization of the enzyme should appear progressively and in parallel with tolerance during chronic ethanol treatment.

The present study was undertaken to clarify the temporal relationship between acquisition of tolerance to ethanol as evidenced by physiological and behavioral measurements on the one hand, and (Na^+ + K^+)ATPase activity and its inhibition by ethanol in the presence of NA on the other. We chose to study the ATPase activity of isolated nerve terminals (synaptosomes) where a large part of the (Na^+ + K^+)ATPase activity of the central nervous system is concentrated.

METHOD

Ethanol Administration

Male Wistar rats weighing 150–180 g were purchased from

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Biobreeding Laboratories (Ottawa, Canada) and individually housed in temperature-controlled rooms. Ethanol treated and control rats were pair-fed with respect to the ration of Purina rat chow and maintained on water ad lib. Ethanol in water solution was administered by intragastric intubation in a constant volume of 24 ml/kg body wt. The initial dose was 3 g of ethanol/kg body wt. daily, and it was increased by 0.5 g/kg every 3 days. For dosages higher than 6 g/kg (i.e., more than 3 weeks treatment) the animals were intubated twice daily with a portion of the total dose, in order to avoid too large a volume of gavage. Control rats were given starch in a dose equicaloric with that of ethanol and in the same volume. ATPase activity was determined after one, two and three weeks of ethanol treatment. Eight ethanol-treated animals and eight pair-fed controls were used for each treatment period.

Tolerance to ethanol was tested after four days, one week, two weeks (six ethanol-treated and six control animals), three weeks (eight ethanol-treated and eight control animals), and four weeks (six ethanol-treated and six control animals). The animals used in the tolerance tests continued to receive their respective ethanol or starch treatments for at least another week before being sacrificed for the enzyme assays. The purpose was to avoid any possible confounding effect of stress, due to the IP injections, on the enzyme activity. Thus, at any given time, different animals were used for the tolerance measurements and for the enzyme assays. On the day of sacrifice, the ethanol-treated and control animals were given by gavage 2 g ethanol/kg or an equicaloric equivalent dose of starch solution respectively, 1 hour before killing, in order to avoid any withdrawal reaction [30,31].

Ethanol Tolerance Tests

Two tests were chosen for assessing the development of tolerance during the ethanol treatment. The tilting plane was used as a behavioral test of the impairment of motor coordination produced by an acute challenge dose of ethanol [2]. This test was preferred to others available because it does not require any training of the animals and therefore does not implicate any learning process [16,37]. The other test was physiological and consisted of the hypothermic response produced by a challenge dose of ethanol.

Tilting plane. The test was carried out with apparatus and procedures similar to those described by Arvola *et al.* [2] but modified by the use of an electric motor and cam to give constant angular velocity of the tilting plane. The plane had a rough surface to which the rats could cling. Each rat in turn was placed on the plane with its head pointed toward the fulcrum, and the angle of the board was increased at a constant rate until the rat was no longer able to maintain its position. The "sliding angle" at which this occurred has been shown to be a good index of intoxication [2,15]. Each rat was tested before any ethanol injection. Ethanol (3 g/kg body wt.) was then given IP as a 17.5% (v/v) solution in saline and the rat was retested every 30 min for 180 min. The sliding angle of the animal on each run during inebriation was calculated as a percentage of its control value. The maximal percentual difference in performance on any of the post-injection runs was used to quantify the motor impairment produced by ethanol. In preliminary studies, 3 g ethanol/kg body wt., IP was found to be necessary to give clear and consistent results in this test.

Body temperature measurement. Body temperature of the rats was recorded at a room temperature of approximately 22°C just before injection of 3 g of ethanol/kg body wt., IP and again every 30 min for 180 min after the injection [19]. A lubricated rectal thermistor probe connected to a Yellow Springs telethermometer (Yellow Springs, OH) was inserted 5 cm into the rectum of the animal for 30 sec or until stable recordings were obtained. The maximal fall in temperature was used to quantify the hypothermic effect of ethanol. The degree of motor impairment on the tilting plane and hypothermia were recorded concurrently after the same injected dose of ethanol.

Preparation of Synaptosomes

The rats were killed by decapitation and the whole brain was removed as quickly as possible, stripped of meninges, wiped clean of blood, weighed, and homogenized at low speed in 10 volumes of ice-cold 0.32 M sucrose containing 5 mM Tris HCl buffer at pH 7.4 in a glass Potter-Elvehjem homogenizer with teflon pestle. Synaptosomes were then prepared from the homegenate according to Cotman and Matthews [6]. The crude mitochondrial pellet was washed three times and then resuspended in 0.32 M sucrose containing 5 mM Tris HCl at pH 7.4, and layered on a discontinuous gradient of 7.5% and 13% Ficoll dissolved in 0.32 M sucrose with 5 mM Tris HCl. After centrifugation for 1.5 hours at 105,000 × g, the synaptosomes were collected at the interface between 7.5 and 13% Ficoll, diluted with 3 volumes of 0.32 M sucrose and 5 mM Tris HCl at pH 7.4, and centrifuged at 35,000 × g for 30 min. All centrifugations were performed at 4°C. The synaptosomal pellet was similarly resuspended once, and finally suspended in 1.4 ml of the same buffer. This suspension was diluted 5-fold with cold distilled water before being assayed for $(Na^+ + K^+)$ATPase.

There was negligible contamination of the synaptosomes by material from other subcellular fractions, as judged by ATPase activities and electron microscopy. Protein concentrations were determined by the method of Lowry *et al.* [26].

$(Na^+ + K^+)$ATPase Assay

The assay mixture for total activity contained 30 mM imidazole, 30 mM glycylglycine, 3 mM Na_2ATP, 120 mM NaCl, 5 mM KCl and 3 mM $MgCl_2$ plus 0.05 ml of the synaptosomal preparation in a final volume of 1.3 ml at 37°C as previously described [30, 31, 32]. In another tube, 1 mM ouabain was added and NaCl and KCl were omitted, for measurement of ouabain-insensitive activity. The reaction was stopped after 20 min by addition of 0.5 ml of 1.2 M $HClO_4$. Inorganic phosphate (Pi) was determined by using ammonium molybdate and 1-amino-2-naphthyl-4-sulphonic acid [18,31]. $(Na^+ + K^+)$ATPase activity was obtained by subtracting the ouabain-insensitive activity from the total activity, and was expressed as micromoles of Pi produced per mg protein per hour. For investigation of the noradrenaline-ethanol interaction, appropriate dilutions of noradrenaline or ethanol were made such that addition of 0.05 ml of each would yield the desired final concentrations in the assay.

Blood Ethanol Determination

Prior to sacrifice, blood was collected from the cut tip of the animal's tail into a capillary tube. An aliquot (0.05 ml) of blood was immediately transferred to a tube containing 1 ml

TABLE 1

EFFECT OF CHRONIC ETHANOL TREATMENT ON THE MAXIMAL CHANGE IN RECTAL TEMPERATURE (Δ T°C) AND MAXIMAL IMPAIRMENT OF PERFORMANCE ON THE TILTING PLANE (%) AFTER AN IP CHALLENGE DOSE OF 3 g OF ETHANOL/kg BODY WEIGHT

Duration of Treatment	N per Group	Δ T°C			Motor Impairment %		
		Starch control	Ethanol	Percent change	Starch control	Ethanol	Percent change
4 days	6	2.25 ± 0.13	2.16 ± 0.13	− 4	31.2 ± 3.0	26.5 ± 1.9	−15
1 week	6	2.42 ± 0.24	2.17 ± 0.21	−10	28.7 ± 2.9	20.5 ± 2.8	−29
2 weeks	6	2.75 ± 0.10	1.50 ± 0.22*	−45	36.4 ± 3.0	19.3 ± 2.5†	−47
3 weeks	8	3.05 ± 0.50	1.80 ± 0.10†	−41	34.0 ± 2.9	17.9 ± 4.3†	−47
4 weeks	6	3.25 ± 0.17	1.50 ± 0.32†	−54	37.5 ± 4.0	17.5 ± 2.9†	−53

The results are mean values ± S.E.M.
p values were determined by Student's t-test for paired data, *$0.01<p\leq0.02$.
†$p\leq0.01$.

of 0.6 M perchloric acid, and ethanol was determined enzymatically in the deproteinized samples [5].

Chemicals

Na_2ATP, 1-arterenol HCl, ouabain, ammonium molybdate, glycylglycine, imidazole, bovine serum albumin and the kit for blood ethanol determinations were obtained from Sigma (St. Louis, MO), Ficoll 400 was purchased from Pharmacia (Uppsala, Sweden), and 1-amino-2-naphthyl-4-sulphonic acid from BDH (Poole, Great Britain). All other reagents were of the highest grade commercially available.

Statistical Analysis

Differences between the control and the ethanol-treated groups were analysed by Student's t-test for paired data. In some instances, two sets of data were compared using linear regression models and correlation coefficients were calculated.

RESULTS

Tests of Tolerance to Ethanol

In spite of the relatively small daily dose of ethanol, the ethanol treatment resulted in a progressive development of tolerance to the motor impairing and hypothermic effects induced by an acute challenge dose of ethanol.

Table 1 shows the effects of the IP test doses of ethanol on motor performance and body temperature in the ethanol-treated and control animals, after 4 days and 1–4 weeks of the respective treatments. The initial body temperature on each test day remained essentially constant through the treatment period. For the first week, the maximal temperature change (ΔT) produced by the test dose of ethanol was the same in the two treatment groups, but after 2–4 weeks it was significantly smaller in the ethanol-treated than in the starch control group. This was due to both a progressive rise of ΔT in the controls and a fall in the ethanol group. The effect on the performance on the tilting plane showed a similar evolution. The degree of tolerance to ethanol was similar for the two tests. There was a positive correlation (r=+0.972) for the intergroup difference on the two tests during the course of the experiment.

TABLE 2

EFFECT OF CHRONIC ETHANOL TREATMENT ON BODY WEIGHT, BRAIN WEIGHT AND PROTEIN CONTENT

Duration of Treatment	Body wt. g	Brain wt. g	Protein mg/g brain
1 week			
Starch	232 ± 7	1.61 ± 0.03	97.28 ± 2.34
Ethanol	235 ± 6	1.57 ± 0.02	101.20 ± 3.30
2 weeks			
Starch	226 ± 10	1.53 ± 0.03	117.20 ± 3.36
Ethanol	224 ± 14	1.59 ± 0.02	111.19 ± 5.61
3 weeks			
Starch	248 ± 6	1.57 ± 0.04	118.16 ± 5.12
Ethanol	248 ± 6	1.60 ± 0.02	111.09 ± 2.90

The values are means ± S.E.M. (N=8 per group). There were no significant differences between the groups.

$(Na^+ + K^+)ATPase$ Assay

After one, two or three weeks of intoxication, the average body and brain weights were essentially the same for the experimental animals and their respective controls. The protein content of whole brain did not differ significantly between the two groups (Table 2).

In the ethanol-treated rats, the blood ethanol concentration at sacrifice time (10:00 a.m.), one hour after administration of 2 g ethanol/kg, was 107±6 mg%. (N=24). There were no significant differences between the levels found after one, two or three weeks of treatment.

As shown in Table 3, the synaptosomal ATPase activity was found to increase slightly but progressively during ethanol treatment. The increase became highly significant after 2 and 3 weeks of treatment (+12% and +18% respectively, compared to the activity in the controls). Moreover, the increase could be correlated with the development of tolerance as assessed by hypothermia (r=+0.778) and motor performance on the tilting plane (r=+0.839).

TABLE 3

EFFECTS OF CHRONIC ETHANOL TREATMENT ON SYNAPTOSOMAL (Na^+ + K^+)ATPase ACTIVITY

Duration of treatment	Starch Control	Ethanol	Percent change
1 week	23.11 ± 1.32	24.69 ± 1.43	+ 7
2 weeks	23.73 ± 0.98	26.54 ± 0.89†	+12
3 weeks	21.46 ± 1.02	25.35 ± 1.79*	+18

The enzyme activities are expressed as μmoles Pi/mg protein/hr, and given as mean values ± S.E.M. (N=8 per group). p values (ethanol vs. controls) were determined by Student's t-test for paired data.

*$0.01<p\leq0.02$.

†$p\leq0.01$.

TABLE 4

EFFECT OF CHRONIC ETHANOL TREATMENT ON NORADRENALINE SENSITIZATION OF SYNAPTOSOMAL (Na^+ + K^+)ATPase TO ETHANOL

Duration of treatment	Starch control	Ethanol	Percent change
1 week	33.3 ± 4.4	31.4 ± 5.3	−5
2 weeks	32.1 ± 2.7	18.5 ± 7.3*	−42
3 weeks	41.1 ± 3.6	19.8 ± 2.2†	−52

The values are expressed as percent inhibition of (Na^+ + K^+)ATPase activity in the presence of 100 mM ethanol and 100 μM noradrenaline, and given as mean values ± S.E.M. (N=8 per group); p values were determined by Student's t-test for paired data.

*$0.01<p\leq0.02$.

†$p\leq0.01$.

Addition *in vitro* of 100 mM ethanol alone, or of 100 μM NA alone, had no effect on the synaptosomal enzyme activity (results not shown). One hundred mM ethanol added *in vitro* in the presence of 100 mM NA caused a similar inhibition of enzyme activity in the ethanol-treated and control rats after one week of treatment. After 2 or 3 weeks of treatment, however, the inhibition was significantly less marked in the ethanol-treated animals (Table 4). The decreased sensitivity of (Na^+ + K^+)ATPase to ethanol added *in vitro* correlated to the development of tolerance as assessed by motor performance (r=+0.660) and by the hypothermic effect of ethanol (r=+0.953). It also correlated to the increase of the basal level of synaptosomal (Na^+ + K^+)ATPase activity (r=+0.931).

DISCUSSION

Although a very large body of literature attests to the fact that changes in brain (Na^+ + K^+)ATPase activity might be an index of ethanol-induced alterations in the neuronal membranes, and might even be a link in a chain of events leading to the production of tolerance, it is impossible to draw a firm conclusion only by retrospective examination of the brains of animals that have already become tolerant to ethanol [34]. We therefore, determined the synaptosomal (Na^+ + K^+)ATPase activity, and its sensitivity to ethanol added *in vitro* during the development of tolerance to ethanol in rats. With the alcohol administration regiment used here, functional tolerance appeared progressively and became statistically significant after two weeks of treatment.

The parameters of tolerance used, i.e. the hypothermic response [20,33] and impairment of motor performance on the tilting plane after an acute challenge dose of ethanol, were found to correlate well with each other and to give parallel temporal results, in spite of their different nature [16]. Simultaneously, we noted a slight but progressively increasing augmentation of the basal synaptosomal (Na^+ + K^+)ATPase. The finding of an increased activity in non-withdrawn animals does not confirm earlier studies [30]. It may be due in part to differences in the experimental paradigm, especially with respect to animal age, use of starch instead of sucrose as carbohydrate source for controls, and intubation of a smaller challenge dose (2 g/kg instead of 3 g/kg) before sacrifice to minimize acute intoxication while avoiding any withdrawal effect. However, we found that in synaptosomal membranes from non-withdrawn ethanol-tolerant rats, the NA-ethanol interaction was markedly reduced in comparison with controls, confirming and extending earlier results concerning the noradrenaline-produced sensitization of ATPase to ethanol [31,32]. It also supports the view that the changes in the ethanol-treated group are accompaniments of tolerance [12] and not consequences of a withdrawal reaction [35]. Moreover, the reduction in the NA-ethanol interaction displayed a time course of appearance which correlated positively with the time course for the development of functional tolerance.

Several mechanisms are possible to explain the changes in (Na^+ + K^+)ATPase activity and sensitivity. The sensitizing effect of noradrenaline occurs through an α-adrenergic receptor-mediated mechanism [17,32]. Although chronic ethanol treatment was not reported to induce any statistically significant changes in the maximal number of α-receptors, their binding affinities were distinctly increased [4]. Increased binding affinity would correlate enhanced stability of the agonist-receptor complex. Coupling between receptor and catalytic units, such as those of (Na^+ + K^+)ATPase, involves conformational changes in the respective proteins. This coupling mechanism could be modified in ethanol-tolerant animals and cause a decreased responsiveness of (Na^+ + K^+)ATPase to noradrenaline and thus to ethanol [31]. Furthermore, it has been suggested, and partially demonstrated, that the facilitation of conformational changes by the α-adrenergic agonist NA is mediated through an enhanced fluidization of membrane lipids [32], perhaps by a mechanism analogous to the α-adrenergic action on adenylate cyclase. This latter action is connected with an increased membrane phospholipid methylation, affecting membrane lipid structure and function as well as lipid-protein interactions [3,9]. Phosphatidylethanolamine and principally phosphatidylserine are normally required for optimal activity of (Na^+ + K^+)ATPase [10,25] and a striking enhancement of the brain phosphatidylserine level [28], as well as disturbances in phosphatidylethanolamine and phosphatidylserine turnovers [22], have been noted after chronic ethanol consumption. The fact that the NA-ethanol interaction on ATPase activity was progressively reduced in membranes from rats made tolerant to ethanol may represent an effect of chronic ethanol treatment on the capacity of the membrane for rapid fluidization by ethanol and NA, i.e., a progressive

change of viscosity of this membrane. In addition, the progressive lack of effect of noradrenaline could be related to a progressive decrease in sialic acid content that we found in the synaptosomes during the chronic ethanol treatment (to be reported separately) in agreement with previous work on young rats [21,36]. Variations of membrane sialic acid content may alter the binding properties of several receptors [27,38]. An effect of ethanol on membrane sialic acid might be another mechanism behind the altered ability of NA to sensitize the $(Na^+ + K^+)$ATPase activity to ethanol inhibition during the development of tolerance to ethanol.

In summary, the present results provide some support for the concept that alterations of $(Na^+ + K^+)$ATPase activity may be mechanistically related to the development of the tolerance to ethanol. Further investigations are needed to explain the underlying mechanisms, and especially the role of ethanol-induced membrane disordering. Nevertheless, the results indicate that the degree of noradrenaline-ethanol interaction with membrane-bound $(Na^+ + K^+)$ATPase could be a quantitative index of tolerance to ethanol.

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