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Inhibition of Plasma Membrane and Mitochondrial Transmembrane Potentials by Ethanol

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The actions of ethanol and its primary oxidative metabolite, acetaldehyde, on plasma membrane and mitochondrial transmembrane potentials were examined in rat brain using fluorescence techniques. Subchronic treatment of adult rats with ethanol resulted in a significant depolarization of both the plasma and mitochondrial membranes when the mean blood ethanol level of the rats was 59 ± 11 mM (mean ± SEM, n = 6). Acute dosing of animals (4.5 g/kg, i.p.) failed to show any significant alterations. Various concentrations of ethanol, added in vitro to a crude synaptosomal preparation isolated from the rat cerebrocortex (P_2) from untreated animals, depolarized both the plasma and mitochondrial transmembrane potentials in a dose-related manner. Addition of acetaldehyde in vitro did not reveal any significant effects on plasma or mitochondrial transmembrane potential.

KEY WORDS: Ethanol; membrane potential; mitochondrial potential; plasma membrane; nervous system; synaptosomes.

INTRODUCTION

The primary focus of this study was to examine the relative potency of ethanol and its primary oxidative metabolite, acetaldehyde, in disruption of membrane potential in the synaptosome. Since it has been shown that a major morphological locus of action of alcohol is the synaptic nerve ending (1,2), all assays were done using P_2, a crude synaptosomal preparation isolated from rat brain.

Transmembrane potentials of nerve terminals modulate both directly and indirectly, a variety of events associated with terminal metabolism and cell-to-cell signaling. The establishment and maintenance of a transmembrane potential is crucial for normal uptake of amino acids and regulation of neurotransmitter release. In turn, the maintenance of the bilayered structure of biological membranes is critical for stabilization of the transmembrane potential. Any damage to the integrity of the membrane may lead to a disruption of the transmembrane potential. Since ethanol has been shown to make the synaptosomal membrane more fluid (3–5), it may modulate plasma membrane potential. A study conducted by Boonstra et al. (6) showed that an increase in the lateral mobility of lipids in the plasma membrane was accompanied by a marked depolarization of the membrane potential.

The literature contains few investigations relevant to the current study. Murphy and Tipton (7) reported that proton leak through the mitochondrial inner membrane was unaffected by ethanol treatment and thus changes in proton leak were not responsible for alterations in respiration found in the mitochondria isolated from ethanol-treated rats. They concluded that the lowered coupled respiration rate which was observed, was solely due to decreased activity of the electron transport chain. In another study assessing mitochondrial transmembrane po-

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tential as an indicator of cytotoxicity, acetaldehyde was not found to reduce mitochondrial potential in rat liver epithelial cells (WB cell line) or human skin fibroblasts (MSU-2 cell line) (8). Since there are very few reports on the effects of ethanol and acetaldehyde upon membrane potentials, their effects on potential across the plasma and mitochondrial membranes have been studied both in dosed animals and in an isolated system.

Experiments were performed using a freshly prepared crude synaptosomal fraction, as membrane depolarization cannot be determined after freezing (9). The small size of synaptosomes renders them intractable to traditional microelectrode impalement techniques normally used to quantify potentials (10). For this reason, it was necessary to calculate membrane potentials using fluorescence techniques. The probe rhodamine 6G was used to estimate alterations in potential. This cationic fluorescent dye is noncytotoxic and can stain mitochondria directly without passage through endocytic vesicles and lysosomes (11). The incorporation of this dye is dependent on the maintenance of an electrochemical potential across the mitochondrial membrane, as described by the Nernst equation (12). In the absence of a mitochondrial transmembrane potential, the dye will not selectively partition into mitochondria (13). Dissipation of the electrochemical potential is indicated by a decrease in the fluorescence of rhodamine (8). Previous investigations have shown rhodamine 6G to also be of value in the study of potential across the limiting membrane of synaptosomes (14).

EXPERIMENTAL PROCEDURE

Animals. 150–175 g male rats (Charles River Breeding Laboratories, Inc. (Wilmington, MA) of the Sprague-Dawley CD strain were used. Animals were housed four per cage with wood chip bedding and maintained on a 12-hour light/dark cycle in a temperature controlled (20 ± 1°C) room. Food (Purina Laboratory Chow, St. Louis, MO) and water were provided ad lib. until the start of the feeding protocol (for in vivo studies only).

Ethanol Treatment

Acute exposure study. Animals were injected i.p. at two sites with a solution of 25% ethanol (v/v) in physiological (0.9% NaCl) saline at a dose of 4.5 g ethanol per kg body weight. They were sacrificed 4 hrs after injection. Control rats were injected i.p. with saline.

Subchronic exposure study. An all liquid nutrient (Sustacal, Mead Johnson, Evansville, IN) was employed. All rats received 88% Sustacal and 12% water (v/v) for 2 days. Then half of the rats received 88% Sustacal, 7% water, and 5% ethanol (v/v) for 2 further days. Fresh solutions were made up daily. At this time, the ethanol proportion was raised to 7% and water was reduced to 5%. The fluid intake of the control group drinking the liquid nutrient alone was limited to 120% of the volume drunk on the previous day by the group receiving the ethanol-containing liquid diet. This was isocaloric with the ethanol-containing nutrient diet. Rat weights were monitored and recorded every second day, together with the fluid consumption of each group. Rates of weight gain were identical in control and alcohol-treated groups. Ethanol consumption averaged 11.9 ± 1.4 g/kg of body wt per day. Animals were decapitated after a total of 10 ethanol drinking days.

Preparation of P_2 Fractions. Rats were decapitated, the brains excised quickly on ice, and the whole brain except the cerebellum and pons-medulla dissected out. Each tissue was weighed, homogenized in 10 volumes of cold 0.32 M sucrose, and centrifuged for 10 min at 1800 × g at 2°C to remove the crude nuclear fraction (P_1). The resulting supernatant fraction (S_1) was centrifuged for 10 min at 31,500 × g to yield the P_2 pellet. This was suspended in HEPES buffer, pH 7.4, to give a tissue concentration of 0.037 g-equivalent/ml (about 0.8 mg/ml of protein). The composition of HEPES buffer was (mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0; and HEPES, 10.

Determination of Transmembrane Potential. 200 µl aliquots of the P_2 suspension were each diluted with 1.6 ml HEPES buffer, pH 7.4, and incubated 5 min at 37°C in the presence of 1 µM rhodamine 6G (15). This concentration of rhodamine has a negligible effect on synaptosomal metabolism (16).

Determination of Mitochondrial Transmembrane Potential. After an initial fluorescent reading was taken at excitation and emission wavelengths of 520 nm and 550 nm, respectively, with the band width of both monochromators at 5 nm, KCl was added to a final concentration of 100 mM in order to depolarize the plasma membrane while not changing mitochondrial potential (14,17). The fluorescence of rhodamine was then determined again. For in vitro studies, ethanol or acetaldehyde was added after this determination. Incubation was continued for a further 10 min at 37°C after which fluorescence was again recorded. In order to completely depolarize mitochondria, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was then added to a final concentration of 1 µM (18) and fluorescence was measured 5 min later.

Determination of Plasma Transmembrane Potential. Plasma membrane potential was estimated in a parallel manner. After initial fluorescence was recorded, 1 µM CCCP was added in order to remove any contribution of mitochondrial potential, and rhodamine fluorescence was measured 5 min after the depolarization of mitochondria. Plasma membrane potential has been shown to remain unaltered for some time after such a suppression of mitochondrial potential (19). For in vitro studies, either ethanol or acetaldehyde was then added and, after a further 10 min incubation at 37°C, fluorescence was determined once more. A final fluorescent value was recorded immediately after depolarization of the plasma membrane with 100 mM KCl. This permitted the proportional reduction of the plasma membrane potential by ethanol or acetaldehyde to be calculated.

The range of ethanol and acetaldehyde concentrations that was used reflect both moderate and maximal physiological levels occurring in blood during ethanol oxidation. Ethanol, acetaldehyde, rhodamine 6G, and KCl were added in water. CCCP was dissolved in dimethylsulfoxide. Control samples received equivalent amounts of all solutions with the exception of ethanol or acetaldehyde, and an equal volume of water was added in place of ethanol or acetaldehyde. Autofluorescence of P_2 fractions, always less than 1%, was recorded and subtracted before calculation of membrane potential.

Calculation of membrane potentials. Plasma membrane potential was derived from the equation:

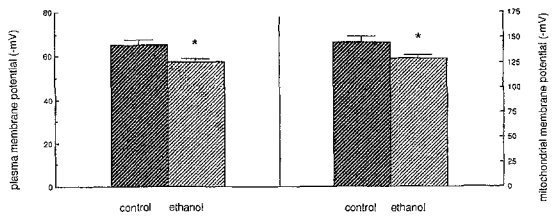

Fig. 1. Effect of ethanol upon plasma and mitochondrial transmembrane potentials in cerebral P_2 fractions isolated from animals 10 days after subchronic administration with an ethanol-containing liquid diet. The control group was isocalorically pair-fed with a corresponding ethanol-free liquid diet. Values were obtained from duplicate determinations per animal with 6 animals/group. Each column represents the mean ± SEM. *Significantly different from corresponding control value ($p < 0.05$).

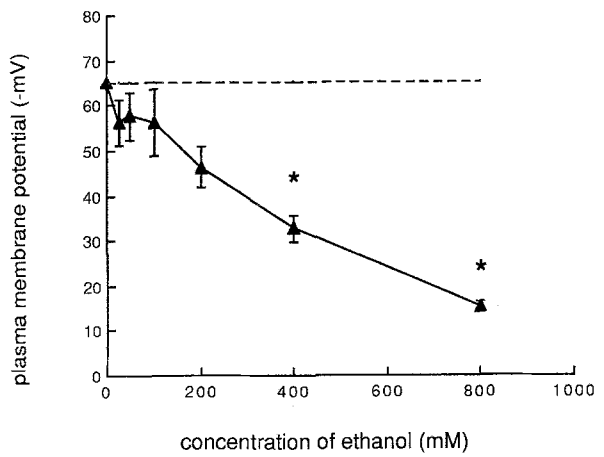

Fig. 2. Effect of varying concentrations of ethanol upon plasma transmembrane potential in cerebral P_2 fractions isolated from untreated animals. Each point represents the mean ± SEM of 3–8 individual replications. *Value differs significantly from control value ($p < 0.05$).

$$(F^{-1} - 1)/(F_k^{-1} - 1) = RT/FE[1 - \exp(-Fc/RT)]$$

where F is the fluorescence of rhodamine when synaptosomes are in HEPES buffer, F_k is that fluorescence in the presence of 100 mM KCl, c is potential, and R, T, and F have the usual thermodynamic significance (12). Mitochondrial potential was similarly calculated substituting F_c (fluorescence in the presence of 1 μM CCCP) for F_k.

Determination of Blood Ethanol. Ethanol was assayed in plasma by enzymatic conversion to acetaldehyde in the presence of NAD. The consequent rise in NADH, which was monitored at 340 nm, is directly proportional to alcohol concentration (20).

Statistical Analysis. Differences between groups were assessed by Fisher's Least Significant Difference Test after one-way analysis of variance. The acceptance level of significance was $p < 0.05$ using a two-tailed distribution.

Chemicals. Ethanol (200 proof) was obtained from Quantum Chemical Corp., USI Division (Tuscola, IL). Acetaldehyde (99%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). KCl was obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals and the ethanol assay kit were obtained from Sigma Chemical Co. (St. Louis, MO).

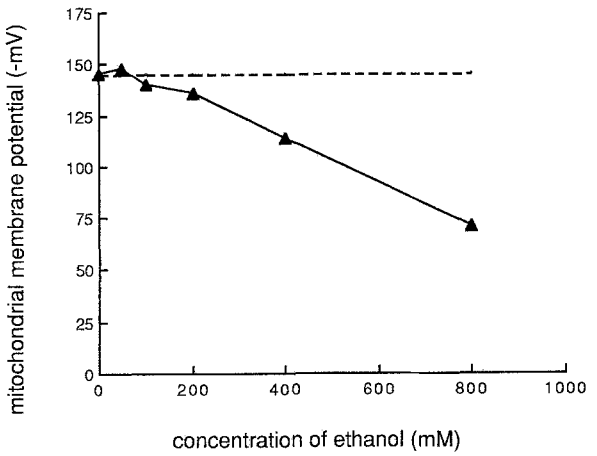

Fig. 3. Effect of varying concentrations of ethanol upon mitochondrial transmembrane potential in cerebral P_2 fractions isolated from untreated animals. Data represent the average from two independent determinations that were within 22% of each other.

RESULTS

Synaptosomal plasma and mitochondrial membrane potentials of control rats were found to have basal values of −65 ± 3 and −145 ± 7 mV at time of sacrifice, respectively (9).

The mean blood ethanol level of rats exposed to the subchronic ethanol regimen for 10 days was 59 ± 11 mM (mean ± SEM, n = 6). This treatment resulted in a significant depression in potential across both the plasma and mitochondrial membranes (Fig. 1). In the rats receiving a single acute dose of ethanol, the mean blood ethanol concentration of the treated rats after 4 hours was 146.3 ± 5.9 mM (n = 6). At this time point, no significant effect on either the plasma membrane or mitochondrial transmembrane potential was found (data not shown).

The effect of various concentrations of ethanol upon plasma and mitochondrial transmembrane potentials in P_2 fractions exposed in vitro was also studied. Plasma membranes were depolarized by ethanol in a dose-dependent manner (Fig. 2). This effect was significant at ethanol concentrations of 400 and 800 mM only. Ethanol also depressed mitochondrial potentials to a lesser extent in a dose-dependant manner (Fig. 3).

In a parallel set of experiments, the role of acetaldehyde on transmembrane potentials was examined in vitro. Exposure of P_2 to different concentrations of acetaldehyde at levels ranging from 25 to 200 μM did not significantly modify plasma or mitochondrial transmembrane potential (data not shown).

DISCUSSION

Ethanol. A prior investigation on the effects of ethanol on Purkinje neurons in rat cerebellar brain slices found that 100 mM ethanol had a minimal effect on membrane potential (21). In the present study, such a concentration of ethanol also had little effect on transmembrane potential in vitro. Another report, using a permeant fluorescent cation in rat synaptosomes, found slight decreases in membrane potential (of 1 to 4 mV) in the presence of 100 to 600 mM ethanol (22). While these changes were not significant, they were in the same direction as our results. Within the last few years, there have been several studies on the electrical properties of cells in isolation using neuronal cell cultures. In cell culture studies using fetal chick and mouse spinal cord neurons, ethanol, at concentrations of up to 100 mM, was found to have little or no significant change in resting membrane potential (23,24). The same was true for cerebellar Purkinje neurons and hippocampal neurons (25). There are however other reports showing biphasic responses (26–28).

Subchronic exposure of rats to ethanol depressed membrane potentials significantly below that of the control value. This parallels our findings in isolated synaptosomes. However, the attenuations found in membrane potentials of synaptosomes isolated from ethanol-treated animals cannot be due to a direct influence of ethanol since this was essentially removed during tissue fractionation. Therefore, the in vivo results reflect permanent changes in membrane structure induced by ethanol. A possible explanation for our findings may be the ethanol-induced depression of the activity of the Na^+/K^+ ATPase. Brodie and Sampson (29) demonstrated that ethanol acutely produced a dose-dependent depolarization of the transmembrane resting potential in cultured skeletal myotubes. Furthermore, they showed that this effect was associated with an inhibitory action of the Na^+/K^+ ATPase pump. These authors concluded that the decrease in resting transmembrane potential by ethanol was a result of inhibition of Na^+/K^+ ATPase activity. Ethanol has been shown to inhibit microsomal Na^+/K^+ ATPase activity in vitro in a concentration-dependent manner in the brains of rats, guinea pigs, and mice, and in the electroplaque of the electric eel (30,31). Significant inhibition of this enzyme was also demonstrated in vitro in hepatocytes after addition of ethanol (32). Several other studies have shown that acute exposure to ethanol depresses the activity of Na^+/K^+ ATPase in a variety of excitable and non-excitable cells (33–36). The Na^+/K^+ ATPase activity appears to be a major factor in the determination of the resting potential of the plasma membrane (37–39).

Ethanol is known to affect several additional membrane-bound and soluble enzymes (33–35). A range of synaptic and other physiological activities of neurons are also affected (23,26,27). Thus, it would be inaccurate to suggest that the depolarizing effects of ethanol on the transmembrane potential of synaptosomes could be unequivocally ascribed to a single action such as the inhibition of the Na^+/K^+ ATPase. Furthermore, the inhibition of the Na^+/K^+ ATPase would be expected to be initially associated with neuronal excitation, not depression. Therefore, it remains unclear how ethanol's alterations of resting transmembrane potential and Na^+/K^+ ATPase activity could contribute to its depressant actions. The potentially excitatory effects of ethanol, as a result of plasma membrane depolarization which were demonstrated in the present study, may be a compensatory reaction for the depressant effects of ethanol related to modulation of ion channels.

The mitochondrial membrane potential was also depressed in a dose-related manner by ethanol added in vitro to synaptosomes (Fig. 3). Murphy and Tipton (7) reported that chronic ethanol treatment decreased the activity of the components of the hepatic mitochondrial electron transport chain or substrate transporters which have control over coupled, but not uncoupled, respiration. Under normal conditions, the transfer of electrons through the respiratory chain leads to the pumping of protons from the matrix to the other side of the inner mitochondrial membrane. Since the transmitochondrial membrane potential is generated by, and dependent upon, this movement of protons across the inner mitochondrial membrane, any alteration in activity of the electron transport chain may modify mitochondrial potential. This, in turn, may have adverse effects on mitochondrial energy generation, in particular, ATP synthesis because this proton gradient is critical for the formation of ATP from ADP. Indirectly, this also has an effect on the plasma membrane potential because maintenance of this gradient is an energy-requiring process.

Acetaldehyde. Examination of the action of in vitro acetaldehyde upon plasma and mitochondrial transmembrane potentials failed to reveal any significant modifications at either of the membranes, even at concentrations in excess of those that have been reported in the brain of the intact ethanol-treated rat, i.e., 5 μM (40) (Table II). Only one previous work is known to have explored the effect of acetaldehyde on mitochondrial transmembrane potential (8). In trying to assess alterations in mitochondrial transmembrane potential as an indicator of cytotoxicity, these researchers found that the addition of very high concentrations of acetaldehyde (7.7, 15.0, and 22.5 mM for 1 hr) to cultured rat liver

epithelial cells (WB cell line) or human skin fibroblasts (MSU-2 cell line) did not reduce the potential in either cell line. Another study which examined in vitro acetaldehyde effects on hepatic plasma membrane ATPases found no significant change in Na^+/K^+ ATPase after addition of 10 to 480 μM and 1 and 3 mM acetaldehyde (32). Following addition of acetaldehyde at concentrations of 6 mM and higher, progressive inhibition was observed. However, such concentrations are very much higher than those occurring in an intact organism during ethanol oxidation. In yet another investigation, acetaldehyde, at concentrations of 1 mM and greater, was found to inhibit mitochondrial respiration, energy production, and energy utilization (41). Again, such high concentrations are physiologically irrelevant. These observations suggest that, at physiological concentrations, acetaldehyde does not affect either the plasma or mitochondrial transmembrane potential.

Taking the results as a whole, it is clear that the effects of ethanol exposure upon membrane potentials are likely to be caused by changes in membrane structure consequent to ethanol catabolism rather than by ethanol directly or by its metabolites.

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