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EFFECTS OF ETHANOL ON ARRHENIUS PARAMETERS AND ACTIVITY OF MOUSE STRIATAL ADENYLATE CYCLASE

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Abstract—Arrhenius plots of basal and dopamine (DA)-stimulated adenylate cyclase activities exhibited discontinuities at 20°, while the plot of fluoride-stimulated adenylate cyclase activity was linear over the studied temperature range. None of the Arrhenius parameters were altered by *in vitro* addition of ethanol (75 or 750 mM) to enzyme assay mixtures, and Arrhenius parameters were found to be unchanged when enzyme obtained from animals rendered tolerant to, and physically dependent on, ethanol was assayed. The differences between the response to ethanol of adenylate cyclase and the response of other membrane-bound enzymes [e.g. (Na^+–K^+)ATPase], as measured by Arrhenius plots, may indicate different sites of action of ethanol. When the specific activity of adenylate cyclase was examined, ethanol was found to stimulate activity at all temperatures tested. The dose–response curve for ethanol activation of basal adenylate cyclase activity was shifted to the right for enzyme obtained from mice chronically treated with ethanol. Analysis of the data indicated that activation of adenylate cyclase by ethanol (as well as by DA) was an entropy-driven process. Since ethanol treatment did not affect the Arrhenius parameters, which appear to be associated with membrane lipids, it is suggested that enzyme activation by ethanol results from direct effects on the enzyme or regulatory protein. Resistance to this effect occurs through changes in protein conformation following chronic ethanol treatment.

The actions of ethanol in the central nervous system (CNS), including the development of functional tolerance and physical dependence, have in recent years often been ascribed to the initial "fluidizing" or disordering effects of ethanol on neuronal membranes [1–3] and to the subsequent adaptive responses which induce resistance to these effects [1, 4–6]. The evidence for this hypothesis comes mainly from investigations of the influence of ethanol on the bulk lipid properties of biological or model membranes [1–3, 7].

It should be kept in mind, however, that although lipids constitute the main structural component of cell membranes, the functional components are membrane-bound proteins, including enzymes and receptors. The activities of many of these proteins are influenced by surrounding lipids [8], but it is not clear how each membrane-bound system will be affected by changes in bulk lipid properties. Furthermore, biological membranes are not homogeneous in terms of lipid composition (e.g. Ref. 9). Synaptosomal membranes, among others, exhibit asymmetry of phospholipid and cholesterol composition, resulting in differences in fluidity between the inner and outer leaflets [9, 10]. For these reasons, overall changes in membrane physical properties following acute or chronic ethanol treatment may be misleading, when interpreted in terms of a site or mechanism of ethanol's action.

Since many neuronal membrane-bound enzymes and receptors have been localized to specific areas of the membrane (e.g. inner or outer leaflet) and, in fact, optimal activity of membrane-bound enzymes appears to require specific phospholipid "microdomains" [9, 11–16], such enzymes can be used as site-specific probes to monitor the response to ethanol exposure in particular areas of the neuronal membrane, as well as in particular cell types. In effect, the sensitivity to ethanol of membrane lipid–protein interactions, rather than bulk lipid properties, can be assessed.

The influence of the lipids immediately surrounding a particular enzyme can, in certain cases, be evaluated by measuring the temperature dependence of the enzyme activity (Arrhenius plot). The curves obtained often exhibit a discontinuity (transition temperature) which, in the case of (Na^+–K^+)ATPase and adenylate cyclase, has been shown to be associated with phase transitions or phase separations of surrounding membrane lipids [8, 17]. We have found previously that the transition temperature of mouse synaptosomal (Na^+–K^+)ATPase, an enzyme which spans the neuronal membrane, is altered by ethanol treatment, and that enzyme obtained from ethanol-tolerant animals is resistant to the effects of ethanol [18]. The present studies were undertaken to evaluate the effect of ethanol treatment on Arrhenius parameters of another membrane-associated enzyme, dopamine (DA)-sensitive adenylate

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cyclase. The catalytic unit of this enzyme resides in the cytoplasmic membrane, while the neurotransmitter receptor is in the outer membrane leaflet [9]. The response of this enzyme activity to ethanol treatment could, therefore, localize changes due to ethanol treatment in particular portions of the neuronal membrane.

METHODS

Male C57B l/6 mice (23 ± 2 g) were housed six to a cage in our laboratory environment (ambient perature, 22 ± 1°; 12-hr light cycle) for at least 7 days with *ad lib.* access to Purina Laboratory Chow and water before the experiments were begun. For experiments which involved the chronic administration of ethanol, animals were housed individually, acclimated for 1 day to a liquid diet consisting of Carnation Slender, sucrose (96.8 g/l) and vitamin supplement (ICN Corp., Cleveland, OH; 3 g/l) and, then, either continued on this diet (controls) or offered a liquid diet containing ethanol U.S.P. (59.6 g/l) as an equicaloric replacement for the sucrose [19]. Daily intake of the sucrose-containing diet by control animals was restricted to the equivalent of the mean caloric intake of the animals drinking the ethanol-containing diet. During the period of ethanol drinking, animals were monitored daily for ethanol consumption and intoxication [19].

After they had consumed the ethanol-containing diet for 7 days, all mice were again given the control diet (withdrawal) and withdrawal symptomatology was monitored as described previously [19, 20].

Striatal adenylate cyclase activity. Animals were decapitated, and the brains were rapidly removed and dissected on a chilled (4°) glass plate. The caudate nuclei were removed as described previously [21]. Tissue obtained from ten to twelve mice was pooled and homogenized at 4° in 10 vol. of 2 mM Tris–maleate buffer, pH 7.4, containing 2 mM ethyleneglycolbis (amino-ethylether) tetra-acetate (EGTA) [21, 22]. The homogenate was centrifuged at 600 *g* for 15 min, the supernatant fraction was decanted, the pellet was resuspended in a small volume of homogenizing buffer, and the suspension was centrifuged at 300 *g* for 15 min. The supernatant fractions were combined and centrifuged at 102,000 *g* for 1 hr. The resulting pellet was resuspended in homogenizing buffer to give a protein concentration of about 2–3 mg/ml [23], the suspension was frozen overnight at −20°, and enzyme activity was assayed the following day.

The standard assay system (pH 7.4; final volume, 0.5 ml) contained MOPS [3-(*N*-morpholino) propanesulfonic acid] (25 mM) as a buffer, since the pH of this buffer was demonstrated to vary only 0.13 pH units over the temperature range used. The assay buffer contained 10 mM theophylline, 0.2 mM EDTA, 10 mM $MgCl_2$, 2 mM [α-^{32}P]ATP (~ 2×10^6 cpm) (New England Nuclear Corp., Boston, MA), and dopamine, fluoride or ethanol at the concentration indicated in the table and figures. The reaction was initiated by addition of the membrane suspension (0.05 ml). Preliminary experiments determined the linearity of the reaction with time at various temperatures, and assays were carried out for 5 min at temperatures of 25° or higher, and for 15 min at temperatures below 25°. In preliminary experiments, it was also determined that the concentration of ATP used was not rate-limiting over the time periods studied, in the presence or absence of dopamine, and that the change in pH over the temperature range used did not measurably alter activity.

The reaction was terminated by immersing the reaction mixture in boiling water for 3 min. After addition of 0.5 ml of a carrier solution containing 8 mM ATP and 0.28 mM [^{3}H]cAMP (~ 20,000 cpm), tubes were centrifuged at 1000 *g* and the supernatant fractions were transferred to fresh tubes and stored at −20°. Cyclic 3′,5′-adenosine monophosphate (cAMP) was isolated from the supernatant fractions as previously described [21], using sequential chromatography on Dowex-50 and alumina columns [24]. The effluents of the alumina columns were added to 3a70b scintillation mixture (RPI, Mount Prospect, IL), and [^{32}P]cAMP was quantitated by liquid scintillation counting. All reported results are adjusted for recovery.

Lines were fitted to the data points in Arrhenius plots by regression analysis, as previously described in our laboratory [18]. Correlation coefficients, slopes and coordinates of intersection of the lines were determined by computer analysis, using a program as described by Korn and Korn [25].

RESULTS

At the time of withdrawal from chronic ethanol treatment, mice were physically dependent on, and tolerant to, ethanol [19]. Withdrawal signs peaked approximately 8 hr after withdrawal. At 24 hr after withdrawal, which is the time at which mice were killed in the present experiments, the animals still displayed tolerance to, and physical dependence on, ethanol, but ethanol had been eliminated, and overt withdrawal signs had dissipated [19, 20].

Arrhenius plots of basal and stimulated striatal adenylate cyclase activity. The temperature-dependence curves of both basal (unstimulated) and DA (10^{-4} M)-stimulated adenylate cyclase activities from C57Bl mouse striatum showed break points at about 20° (Fig. 1). There was no difference in the transition temperature of either of these activities when enzyme from control animals was compared to enzyme obtained from animals withdrawn from chronic ethanol treatment (Fig. 1 and Table 1). The energies of activation (E_A) for basal and DA-stimulated striatal adenylate cyclase, calculated from the slopes of the regression lines, were different above and below the transition temperature, but did not differ significantly between control and ethanol-treated animals (Table 1). Fluoride-stimulated striatal adenylate cyclase activity did not demonstrate a transition temperature over the temperature range studied, and the E_A of enzyme from ethanol-treated mice was not significantly different from that of controls (Table 1).

In terms of the response of adenylate cyclase to activators, as was previously reported for striatal homogenates [21], enzyme from ethanol-treated animals generally showed a decreased response to DA, as compared to that from controls, at all tem-

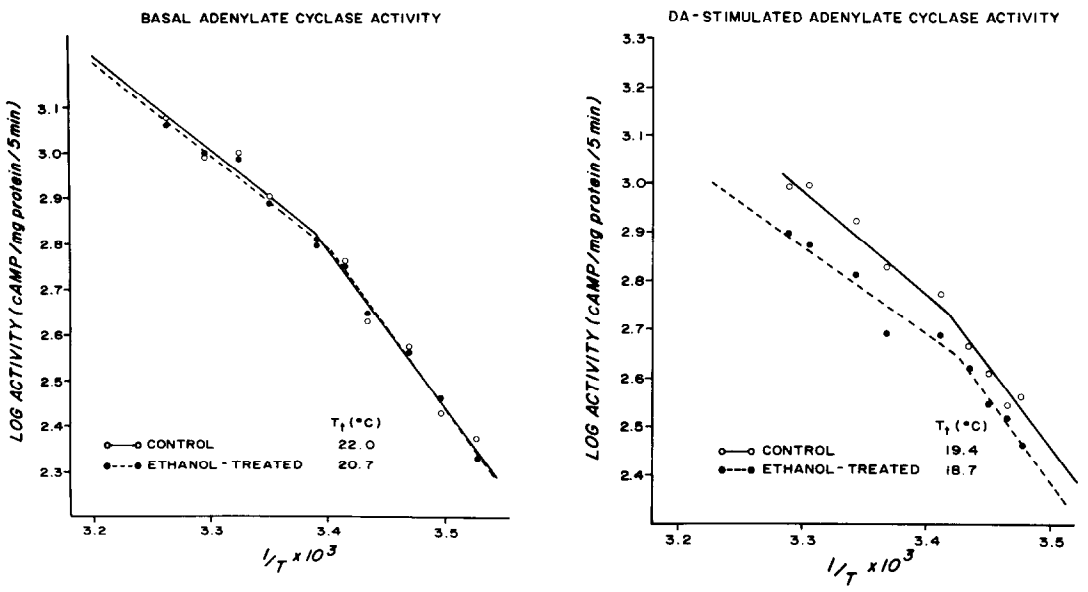

Fig. 1. Arrhenius plots of basal (left panel) and DA-stimulated (right panel) striatal adenylate cyclase activities. Striatal membranes were obtained from control mice (○) and mice that had been chronically treated with ethanol and withdrawn for 24 hr (●). Adenylate cyclase activity, in the absence or presence of 10^{-4} M dopamine, was measured over a temperature range of 15–37°. For details of membrane preparation and enzyme assay, see text. The transition temperatures and energies of activation were determined from lines obtained by regression analysis. This figure represents the results of a typical experiment.

peratures tested (Fig. 1). There was no difference in the response to fluoride [21] of enzyme obtained from control animals as compared to ethanol-treated mice.

Response of adenylate cyclase activity to ethanol added in vitro. The addition of low (75 mM) or high (750 mM) concentrations of ethanol to the adenylate cyclase assay system did not affect the transition temperatures of basal enzyme activity from control or ethanol-treated animals. The data obtained using 750 mM ethanol are shown in Table 1. Although 750 mM ethanol tended to increase both E_{A_1} and E_{A_2} in the ethanol-treated and in the control animals, these changes were not statistically significant (Table 1). Experiments with control mice showed that 750 mM ethanol did not affect either the transition temperature or activation energies of DA-stimulated adenylate cyclase activity (T_t, 19.8 ± 0.8; E_{A_1}, 10.7 ± 1.4; E_{A_2}, 16.3 ± 3.0, N = 3); therefore, these experiments were not carried out using enzyme from ethanol-treated animals. These results contrasted with our previous finding that high concentrations of ethanol altered the Arrhenius parameters of brain (Na^+–K^+)ATPase activity [18].

Table 1. Temperature dependence of mouse striatal adenylate cyclase*

	T_t (°)	E_{A_1} (kcal/mole) (above T_t)	E_{A_2} (kcal/mole) (below T_t)	N
Control				
Basal	21.9 ± 1.1	8.8 ± 0.8	20.1 ± 1.8	9
+DA (10^{-4} M)	20.9 ± 0.6	9.8 ± 0.9	19.3 ± 1.8	3
+EtOH (750 mM)	20.4 ± 2.0	9.8 ± 0.6	24.0 ± 0.6	3
+NaF (20 mM)		10.6 ± 0.6		3
Ethanol-treated				
Basal	20.3 ± 0.8	9.1 ± 0.6	17.8 ± 1.0	9
+DA (10^{-4} M)	19.0 ± 0.7	11.3 ± 1.7	17.2 ± 0.5	3
+EtOH (750 mM)	19.1 ± 0.5	11.5 ± 1.8	20.9 ± 0.8	3
+NaF (20 mM)		8.8 ± 0.8		3

* Ethanol-treated C57Bl mice were withdrawn from chronic ethanol treatment for 24 hr. Assays in the presence or absence of stimulators or ethanol were run simultaneously. In the presence of NaF, no transition temperature was observed, and only one E_A value could be calculated. Values are mean ± S.E.M.; N = number of experiments. Comparisons were made between basal and DA-, ethanol- or fluoride-stimulated activities within each group. Each activity was also compared between control and ethanol-treated groups. T_t values were compared by Student's *t*-test and E_A values by confidence limits of the regression lines. Abbreviations: T_t = transition temperature; E_A = activation energy; and EtOH = ethanol.

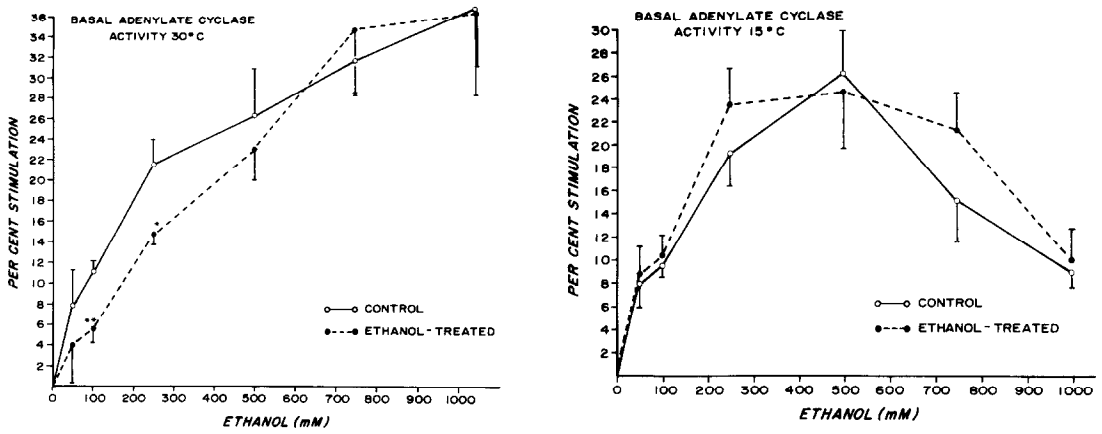

Fig. 2. Activation of striatal adenylate cyclase activity by ethanol added *in vitro*. Basal adenylate cyclase activity in striatal membranes from control mice (○) and mice withdrawn from chronic ethanol treatment for 24 hr (●) was assayed, according to the methods described in the text, in the absence or presence of the indicated concentrations of ethanol. The assay temperature was 30° (left panel) or 15° (right panel). Data are presented as mean ± S.E.M. per cent increase over activity in the absence of ethanol. Asterisks indicate significant differences in per cent stimulation of enzyme from ethanol-treated animals as compared to controls [*t*-test, (*) $P < 0.05$; (**) $P < 0.01$].

In the course of the present experiments, we further examined the *in vitro* stimulation of both basal and DA-stimulated adenylate cyclase activity by ethanol. Such activation had been reported previously by ourselves [21] and others [26], using striatal homogenates. To determine whether tolerance to the activating effect occurred following chronic ethanol administration, dose–response curves for ethanol activation of basal and DA-stimulated activities in control and ethanol-treated animals were constructed (Fig. 2). At 30°, there was a shift to the right of the ethanol dose–response curve for basal enzyme activity from ethanol-treated animals, as compared to controls. The dose–response curve at a temperature *below* the transition temperature, i.e. 15°, followed a pattern different from that at 30°. At 15°, there was no difference in ethanol-induced activation of enzyme between control and ethanol-treated mice. Similar dose–response curves were obtained for ethanol activation of DA-stimulated adenylate cyclase activity at both temperatures. At 30°, the dose–response curve for ethanol activation of DA-stimulated activity showed a shift to the right, as compared to that for control animals, similar to the results for basal activity. However, the differences in the response to ethanol of DA-stimulated activity from control and ethanol-treated animals did not reach statistical significance (data not shown).

DISCUSSION

For many membrane-bound enzymes, the transition temperature determined from Arrhenius plots appears to be associated with phase transitions of the lipids surrounding the enzyme. Direct evidence for this assumption, with regard to (Na^+–K^+)ATPase, has been obtained using physical techniques (electron paramagnetic resonance, EPR) [27]. The mobility of a lipid spin label bound to the enzyme was shown to exhibit the same temperature dependence as enzyme activity [27]. In addition, a change in membrane lipid composition shifted the transition temperature of the ATPase, and in membranes with high cholesterol content it was reported that no transition occurred [17, 28]. Several studies have also shown that alterations in membrane phospholipid composition affect the transition temperature of hormone-stimulated adenylate cyclase from various tissues [14, 29]. These findings are in line with the contention that the transition temperature of adenylate cyclase activity is also associated with changes in the surrounding lipids.

In the present study, both basal and DA-stimulated adenylate cyclase activities from C57Bl mouse striatum exhibited discontinuities in their Arrhenius plots (Fig. 1). Similar results have been reported for epinephrine-stimulated adenylate cyclase from rat brain cortex [30]. On the other hand, fluoride-stimulated adenylate cyclase activity showed a linear temperature dependence over the range studied. Arrhenius plots of adenylate cyclase activities from several peripheral tissues have been constructed previously [29–33]. Basal activity often shows a discontinuous plot (athough this is not always true; [29]), and linear plots are often obtained when fluoride ion is added to the assay [29–31, 33].

In contrast to our previous findings with mouse synaptosomal (Na^+–K^+)ATPase, the transition temperature of striatal adenylate cyclase was not altered significantly by high concentrations of ethanol. The lack of effect was observed both with basal (Table 1) and with DA-stimulated enzyme activities. This result was somewhat surprising, since concentrations of ethanol similar to those that we added *in vitro* can be clearly shown to have disorganizing effects on biological and model membranes [1, 7] and, at the same concentration, ethanol did alter the tran-

sition temperature of synaptosomal (Na^+–K^+)ATPase [18]. The difference between the response of (Na^+–K^+)ATPase [18] and adenylate cyclase to ethanol treatment cannot be totally attributed to a different localization of the enzymes within the membrane. Thus, no ethanol-induced changes were observed either for basal adenylate cyclase activity (reflecting activity of an enzyme located in the inner membrane leaflet) or for DA-stimulated adenylate cyclase activity (reflecting the interaction between receptor located in the outer membrane leaflet and the catalytic unit). In line with the *in vitro* results, there were also no differences in transition temperature when enzyme obtained from mice that had been rendered tolerant to ethanol was compared to enzyme from control mice (Fig. 1 and Table 1).

These results suggest that the lipids surrounding the striatal adenylate cyclase, in contrast to those influencing (Na^+–K^+)ATPase activity, do not alter their properties following acute or chronic ethanol treatment. As discussed above, various membrane-bound enzymes require specific phospholipid microdomains for optimal activity [9, 11–16]. It has been demonstrated that the membrane response to various perturbing agents depends on phospholipid composition and cholesterol content [8, 34]. Very recently, it was reported that the "fluidizing" effect of ethanol is most pronounced in those areas of membranes which, under baseline conditions, are most disordered [35]. Therefore, it is reasonable to expect that particular membrane-bound enzymes will be affected to different degrees by ethanol and, in fact, that the functional response of membranes to ethanol may be most accurately determined by investigating the activity of membrane-bound enzymes.

In the present study, ethanol was found to activate striatal adenylate cyclase activity, and enzyme from chronically ethanol-treated mice was resistant to the activation. Ethanol added *in vitro* has been shown previously to stimulate the activity of a number of adenylate cyclase preparations [36–41], including C57Bl mouse striatal DA-sensitive adenylate cyclase [21, 26]. The mechanism by which ethanol increases both basal and DA-stimulated striatal adenylate cyclase activities has been investigated in some detail [26]. This activation was reversible, and was not due to release of endogenous neurotransmitter, since it was not affected by DA receptor antagonists [26]. The effect of ethanol has also been shown not to result from changes in DA receptor properties or in the degree of coupling of receptor to enzyme (i.e. ethanol did not alter the change in agonist affinity caused by GTP) [26]. The conclusion from this study was that ethanol may modify the interactions between the catalytic and regulatory subunits of the adenylate cyclase, secondary to effects on membrane fluidity [26]. The activation by ethanol of adenylate cyclases from other tissues has also been postulated to be mediated by alterations in membrane fluidity [36, 38, 40]. However, our results (discussed above) do not support the hypothesis that ethanol activates striatal adenylate cyclase activity by an effect on the lipids surrounding this enzyme.

Further insight into the mechanism of activation of adenylate cyclase may be gained by examining thermodynamic parameters [42]. The enthalpy of activation can be obtained from the Arrhenius plot by the equation: $\Delta H\dagger = E_A\text{-}RT$. The free energy of activation, $\Delta G\dagger$, is equal to $\Delta H\dagger - T\Delta S\dagger$, and is related to the rate constant of the reaction, k_r, by the equation: $\Delta G\dagger = -RT\ln k_r + (RT\ln kT/h)$, where "$k$" is Boltzmann's constant, and "h" is Planck's constant. Thus, the rate of the reaction is dependent on both the enthalpy (ΔH) and entropy (ΔS) of activation. With no change in entropy of activation, a decrease in the enthalpy of activation (or, in our experiments, a decrease in E_A) is associated with an increased rate of reaction. These conditions hold for the activation of adenylate cyclase activity by fluoride ion, since stimulation of activity by fluoride was accompanied by a *decrease* of E_{A_2} (Table 1). However, stimulation by DA and by ethanol involved an increased rate of reaction with either no change, or a slight increase in E_A. To allow for the increase in reaction rate with no change in E_A, there must be an increase in $\Delta S\dagger$ (entropy must become less negative). Thus, the activation of striatal adenylate cyclase by DA and by ethanol are entropy-driven processes.

The physical interpretation of thermodynamic parameters in a complex biological system is necessarily difficult. However, it has been postulated [42] that increased adenylate cyclase activity which is accompanied by an increased entropy of activation involves stabilization of the enzyme in a more reactive conformation, but one which restricts the conformational changes required for activity. This results in an increased enthalpy of activation, which is compensated for by the increase in entropy of activation. In the study of Sinensky *et al.* [42], activation of adenylate cyclase was associated with increased acyl chain ordering. However, activation of adenylate cyclase in other systems has been associated with increased membrane "fluidity" [43].

Our results suggest that ethanol, as well as DA, may place the striatal adenylate cyclase in an activated conformation. Ethanol appears to alter enzyme conformation in such a way that activity is increased, and that formation of the active state of the enzyme involves a change to a relatively more disordered conformation (entropy of activation is increased) (cf. Ref. 42). However, since neither low, physiologically attainable, nor high concentrations of ethanol altered the transition temperature of striatal adenylate cyclase, the change in enzyme conformation induced by ethanol most probably does *not* occur via an ethanol–lipid interaction, but rather by a direct effect of ethanol on the enzyme protein, or on enzyme protein–regulatory protein interaction.

Following chronic ethanol treatment, there was no change in absolute adenylate cyclase activity, but there was a decreased response to ethanol (as well as to DA; [21]). The resistance to the effects of ethanol was a temperature-dependent phenomenon (Fig. 2). It is likely that the adenylate cyclase exists in different conformations above and below its transition temperature, due to the influence of the surrounding lipids. While ethanol *in vitro* can activate adenylate cyclase at all temperatures, the postulated change in protein conformation, which occurs fol-

lowing chronic ethanol exposure (also, see Ref. 44), and which renders the enzyme resistant to the effects of ethanol, is only apparent at "physiological" temperatures, i.e. those above the transition temperature. This change in enzyme properties, resulting in a decreased response of the enzyme activity to ethanol, may contribute to the development of tolerance to this drug.

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