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RELATIONSHIP BETWEEN ETHANOL-INDUCED ALTERATIONS IN FLUORESCENCE ANISOTROPY AND ADENYLATE CYCLASE ACTIVITY*

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Abstract—The effects of butanol, ethanol, and ketamine on adenylate cyclase activity and fluorescence anisotropy were determined in membranes prepared from L6 cells. The experiments were designed to test the hypothesis that the effects of ethanol on adenylate cyclase activity are a consequence of ethanol-induced changes in bulk membrane order. Butanol and ethanol elicited concentration-dependent increases in adenylate cyclase activity and caused decreases in the fluorescence anisotropy of diphenylhexatriene. Butanol was more potent than ethanol in reducing fluorescence anisotropy, and it elicited a greater reduction in fluorescence anisotropy than did ethanol. Butanol was also more potent than ethanol in activating adenylate cyclase, but the highest concentration of butanol used caused a smaller increase in enzyme activity than did the highest concentration of ethanol. When the percent change in adenylate cyclase activity was plotted against the percent change in fluorescence anisotropy at each concentration of alcohol, the increase in isoproterenol-stimulated adenylate cyclase activity per unit change in fluorescence polarization was greater with ethanol than with butanol. Ketamine decreased fluorescence anisotropy but, unlike the alcohols, ketamine caused a decrease in adenylate cyclase activity. A reduction in assay temperature attenuated both the ethanol-induced activation of adenylate cyclase activity and the ethanol-induced reduction in fluorescence anisotropy. Although the data are consistent with the theory that ethanol acts upon a hydrophobic region of the membrane to enhance adenylate cyclase activity, activation of the enzyme does not appear to be a consequence of a decrease in bulk membrane order.

Ethanol appears to exert its biological effects through nonspecific interactions with hydrophobic regions of the membrane [1]. Short-chain alcohols alter a number of membrane properties including the gel-to-liquid crystal transition of lipid bilayers [2], the membrane area of erythrocyte ghosts [1], and both the membrane order [3] and fluorescence anisotropy [4] of synaptosomal membranes. The activities of membrane-bound enzymes, such as adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], are also altered by alcohols [5–10]. Ethanol increases basal adenylate cyclase activity and synergistically increases hormone- and NaF-stimulated activities [8, 9]. Activation of adenylate cyclase by ethanol appears to result from an effect on both the regulatory and the catalytic subunits of the enzyme [9, 10]. The lipid solubility of a series of short-chain alcohols correlates with the potency of these agents both to increase adenylate cyclase activity [7, 10, 11] and to decrease bulk membrane order [12]. These findings have led to the hypothesis that activation of adenylate cyclase by short-chain alcohols is a consequence of an alcohol-induced decrease in bulk membrane order [13–15].

The relationship between bulk membrane order and adenylate cyclase activity is, however, unclear. When the membrane order of a 25-hydroxycholesterol-resistant mutant of Chinese hamster ovary cells was varied by the addition of increasing concentrations of cholesterol to growth medium containing delipidated serum, it was observed that increases in basal, prostaglandin E_1-, and fluoride-stimulated adenylate cyclase activities correlated with increases in membrane order [16]. On the other hand, increasing the cholesterol content of platelets by incubation with cholesterol-rich liposomes, which increases membrane order [17], resulted in an increase in basal activity, but decreased prostaglandin E_1- and fluoride-stimulated activities [18]. The use of other compounds which alter membrane order has led, in some cases, to observations of a direct relationship between adenylate cyclase activity and membrane order [19, 20]. Other investigators, however, have failed to observe this relationship [13, 21, 22].

The present study was undertaken to determine the role of ethanol-induced changes in membrane properties in the activation of adenylate cyclase by

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directly comparing the effects of ethanol and several other compounds on the fluorescence anisotropy of DPH* with their effects on adenylate cyclase activity.

MATERIALS AND METHODS

A nonfusing variant of the L6 muscle cell line originally developed by Yaffe [23] was used. Cells were grown on 100-mm Falcon plates in Dulbecco's modification of Eagle's medium with 10% fetal calf serum (Sterile Systems, Inc., Logan, UT) in an atmosphere of 90% air, 10% CO_2 at 37°. Cells were plated at 15,000–20,000 cells/cm^3, fed on day 2, and either subcultured on day 3 using 0.1% trypsin in phosphate-buffered saline or harvested on day 5–6. Cells, harvested after being rinsed three times in cold 2 mM HEPES (pH 7.5), were osmotically shocked by incubation at 4° in cold 2 mM HEPES (pH 7.5) for 15 min. Cells were removed from the culture dishes by scraping with a rubber policeman, and the lysate was centrifuged at 20,000 g for 15 min. The resulting pellet was resuspended in 2 mM HEPES and again centrifuged at 20,000 g for 15 min. This procedure was repeated and the final pellet was resuspended in 2 mM HEPES (pH 7.5).

Adenylate cyclase activity was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP. The [α-^{32}P]ATP was synthesized by the method of Johnson and Walseth [24]. Reactions were carried out in a final volume of 150 μl containing 50 mM HEPES (pH 7.5), 5 mM cAMP, 1 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine, 0.5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.25 mM ATP (containing 1–2 million cpm [α-^{32}P]ATP), 10 mM creatine phosphate, 0.1 mg/ml of creatine kinase, and 50 μM GTP. Reactions were initiated by addition of tissue (40–70 μg of protein in 50 μl) and were carried out for 8 min at 37°, 10 min at 27°, or 15 min at 17°. Incubations were terminated by the addition of 100 μl of a solution containing 50 mM Tris (pH 7.5), 5 mM ATP, and 10% sodium dodecyl sulfate. Reaction tubes were then placed in boiling water for 10–15 min, [^{3}H]cAMP (20,000 cpm) was added to all tubes to determine recovery, and sample volumes were increased to 1 ml with deionized water. A modification [9] of the method of Salomon *et al.* [25] was used to isolate the [^{32}P]cAMP.

Steady-state fluorescence anisotropy of DPH was measured using an SLM model 8000 spectrofluorometer (SLM Instruments, Urbana-Champaign, IL) with a temperature-controlled sample cavity. DPH was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and recrystallized from ethanol. Plasma membranes from L6 cells isolated by the method of Lutton *et al.* [26] were labeled with DPH as follows: membranes (0.1 mg protein/ml in 20 mM HEPES, pH 7.5) were incubated with 1.0 μM DPH (taken from a 1.0-mM stock solution in tetrahydrofuran) at 37° for 40 min with continuous stirring. This was found to be the minimum time required to reach a steady level of fluorescence intensity. Membrane and probe concentrations were shown to be low enough to avoid depolarization artifacts and probe–probe interactions respectively. After labeling, the membranes were diluted with cold buffer, centrifuged at 30,000 g for 30 min, resuspended in the original volume of buffer, and dispensed in 2.0-ml aliquots. Probe molecules were excited using vertically polarized light with a wavelength of 360 nm, and the fluorescence intensity at a wavelength of 429 nm was measured at an angle of 90° to the excitation beam. The components of the fluorescence that were parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of the vertically polarized excitation light were determined by measuring the emitted light through polarizers oriented vertically and horizontally. The fluorescence anisotropy (r), which is inversely related to membrane fluidity, was calculated using the equation:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

Correction was made for differences in the efficiency of transmitting vertically and horizontally polarized light by the emission monochromator by determining the polarization ratio ($I_\parallel/I_\perp$) using horizontally rather than vertically polarized excitation light.

The anisotropy of each 2.0-ml sample was first determined in the absence of alcohol after equilibration at 37° for 5 min. Small volumes of 100% alcohol were added to achieve the desired final concentration, the sample was mixed, and the anisotropy determined. In this way, the dependence of the anisotropy on the entire range of alcohol concentrations was determined for each sample. The fluorescence lifetime of DPH was not affected by the highest concentrations of ethanol, butanol, or ketamine used in these experiments. Ketamine was obtained from Parke–Davis (Morris Plains, NJ).

Protein content was determined by the method of Lowry *et al.* [27] using bovine serum albumin (Fraction V) as a standard.

RESULTS

Addition of ethanol or butanol to membranes prepared from L6 cells resulted in a dose-dependent decrease in the steady-state fluorescence anisotropy of DPH, which was approximately linear with alcohol concentration up to 1.0 M for ethanol and 0.25 M for butanol (Fig. 1). Butanol was more potent than ethanol, which correlates with the greater lipid solubility of butanol. Although it was not possible to determine the maximum effect (efficacy) of butanol due to its limited solubility in water, the maximal effect of butanol on anisotropy was greater than that of ethanol. Similarly, both alcohols elicited dose-dependent increases in isoproterenol-stimulated adenylate cyclase activity (Fig. 2). Butanol was more potent than was ethanol in enhancing enzyme activity but, unlike the effect on anisotropy, the highest concentration of ethanol (which had less effect on anisotropy than did the highest concentration of butanol) elicited a greater increase in adenylate cyclase activity than did the highest concentration of butanol. The percent change in anisotropy at each concentration of alcohol (from Fig. 1) was plotted

* Abbreviations: DPH, diphenylhexatriene; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and cAMP, cyclic AMP, adenosine 3′:5′-cyclic monophosphate.

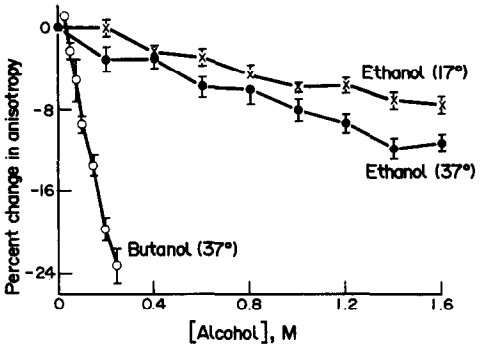

Fig. 1. Effect of short-chain alcohols on fluorescence anisotropy. Fluorescence anisotropy was determined at 37° (○, ●) or 17° (×) in the presence of various concentrations of butanol (○) or ethanol (●, ×). Anisotropy is expressed as the percent change relative to the anisotropy measured in the absence of alcohol. Data are plotted as mean ± S.E.M. for three (butanol) or eight and ten (ethanol) separate experiments.

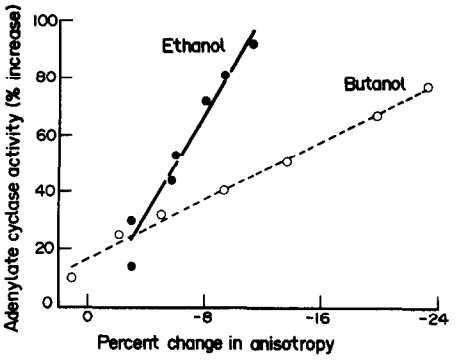

Fig. 3. Relationship between alcohol-induced changes in fluorescence anisotropy and activation of adenylate cyclase. At each concentration of alcohol, the mean value for percent change in anisotropy is plotted against the mean value for adenylate cyclase activity. Values obtained in the absence of alcohol are excluded. These data were taken from experiments shown in Figs. 1 and 2. The product-moment correlation coefficient was −0.979 for the data obtained in the presence of ethanol and −0.996 for the data obtained in the presence of butanol.

against the percent increase in isoproterenol-stimulated adenylate cyclase activity measured at the same concentration of alcohol (from Fig. 2). There was a good correlation between the alcohol-induced decrease in fluorescence anisotropy and activation of adenylate cyclase for both butanol and ethanol (Fig. 3: $r^2 = -0.996$ for butanol and -0.979 for ethanol). However, the increase in isoproterenol-stimulated enzyme activity per unit change in fluorescence anisotropy was greater in the presence of ethanol than in the presence of butanol.

The effect of changes in fluorescence anisotropy on adenylate cyclase activity was investigated further using other agents known to alter the physical properties of the lipid bilayer. Addition of ketamine to membranes prepared from L6 cells decreased fluorescence anisotropy but, in contrast to the effects observed with short-chain alcohols, this decrease was associated with a reduction in isoproterenol-stimulated enzyme activity (Fig. 4). Although phenobarbital has been reported to decrease membrane order and increase adenylate cyclase activity in hepatic membranes [28], no significant effect on either fluorescence anisotropy or isoproterenol-stimulated enzyme activity was observed in L6 membranes incubated with up to 10 mM phenobarbital (data not shown).

The role of alcohol-induced changes in fluorescence anisotropy in the activation of adenylate cyclase activity was investigated further by varying the incubation temperature of the assay. A decrease in incubation temperature attenuated the ethanol-

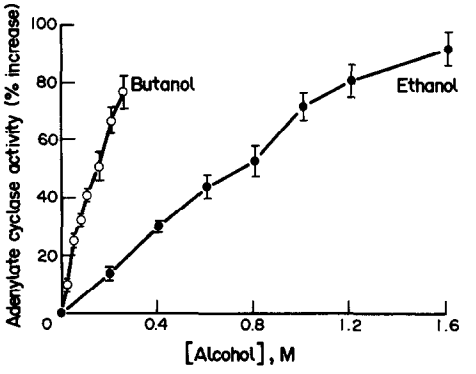

Fig. 2. Alcohol-induced changes in adenylate cyclase activity. Isoproterenol-stimulated (50 μM) adenylate cyclase activity was measured at 37° in the presence of various concentrations of butanol (○) or ethanol (●). Enzyme activity is expressed as a percent of the activity measured in the absence of alcohol. Enzyme activity in the absence and presence of isoproterenol was 102 ± 17.5 and 411 ± 39.9 pmoles/min/mg protein respectively. Data are plotted as mean ± S.E.M. for five separate experiments.

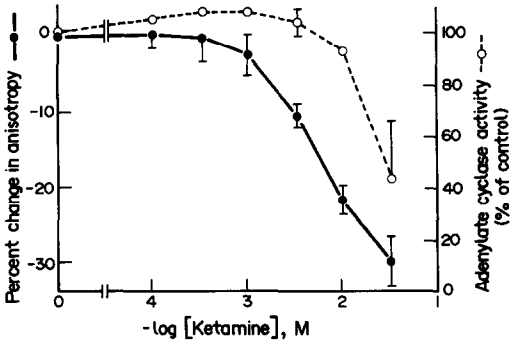

Fig. 4. Effect of ketamine or fluorescence anisotropy and adenylate cyclase activity. Fluorescence anisotropy (●) and isoproterenol-stimulated (50 μM) adenylate cyclase activity (○) were determined in the presence of various concentrations of ketamine. Anisotropy is expressed as the percent change relative to the anisotropy measured in the absence of ketamine. Enzyme activity is expressed as a percent of activity measured in the absence of ketamine (297 ± 33.4 pmoles/min/mg protein). Data are plotted as mean ± S.E.M. for two or three separate experiments.

induced decrease in anisotropy (Fig. 1) and reduced the ability of ethanol to increase isoproterenol-stimulated adenylate cyclase activity (Fig. 5). The slopes of the dose–response plots for ethanol were 40.5 ± 3.1, 111.2 ± 7.8, and 175.2 ± 12.9 pmoles cAMP/min/mg protein/M ethanol at 17°, 27°, and 37° respectively.

DISCUSSION

The activities of membrane-bound enzymes, such as adenylate cyclase, are sensitive to changes in the physical state of the lipid microenvironment [29]. Since alcohols change bulk membrane order, alcohol-induced activation of adenylate cyclase may be a consequence of drug-induced alterations in the order of the lipid bilayer [13–15]. In membranes prepared from L6 cells, short-chain alcohols decreased the fluorescence anisotropy of DPH, a measure of the bulk order of the membrane, and increased adenylate cyclase activity over similar ranges of concentrations. In addition, a decrease in temperature attenuated both the ethanol-induced reduction in fluorescence anisotropy and the ethanol-induced activation of adenylate cyclase. However, if activation of adenylate cyclase is a consequence of the decrease in bulk membrane order, similar changes in fluorescence anisotropy should result in comparable changes in enzyme activity; this was not the case. Over the ranges of concentrations tested, butanol elicited a greater decrease in fluorescence anisotropy than did ethanol, whereas the opposite was true for enzyme activation. Thus, although approximately linear relationships were observed between the percent decrease in fluorescence anisotropy and the percent increase in adenylate cyclase activity for both alcohols, a 3-fold greater activation of adenylate cyclase was found per unit change in anisotropy in the presence of ethanol than in the presence of butanol. In other words, even though the efficacy of butanol could not be determined, over the range of concentrations tested, there was a quantitative discrepancy between the concentration dependence of these two effects.

A further distinction between changes in fluorescence anisotropy and changes in enzyme activity was observed when assays were carried out in the presence of ketamine. Ketamine decreased fluorescence anisotropy in membranes prepared from L6 cells, but this decrease was associated with a reduction rather than an increase in enzyme activity. Okuda *et al.* [20] reported similar effects of ketamine on adenylate cyclase activity and membrane order in rat brain. Ketamine (0.1 or 0.5 mM) decreased NaF- and guanyl-5′-yl-imidodiphosphate-stimulated adenylate cyclase activities and, in the range 0.1 to 10 mM, decreased the fluorescence polarization of DPH. The relationship between the changes in fluorescence anisotropy and enzyme activity observed with ketamine could be explained by postulating a direct inhibitory action on adenylate cyclase, but no evidence exists to support this assumption. The discrepancy between the abilities of ethanol and butanol to affect adenylate cyclase activity and fluorescence anisotropy is probably not due to a direct inhibitory effect of butanol on the enzyme, because in rat striatum there is a linear relationship between activation of the enzyme by short-chain alcohols and the length of the aliphatic chain [10].

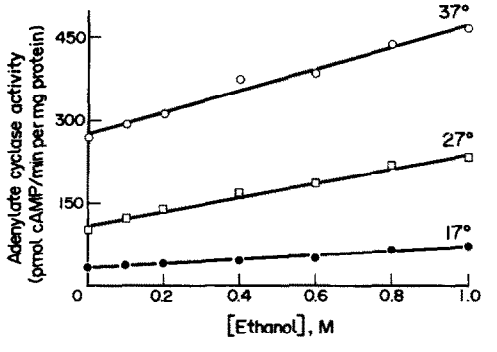

Fig. 5. Effect of temperature on ethanol-induced activation of adenylate cyclase. Isoproterenol-stimulated (50 μM) adenylate cyclase activity was measured at 17° (●), 27° (□), and 37° (○) in the presence of various concentrations of ethanol. Data are representative of four to six separate experiments.

The present finding that ethanol-induced activation of adenylate cyclase is not simply a consequence of a reduction in bulk membrane order may help to explain the many conflicting reports on the association between bulk membrane order and adenylate cyclase activity which appear in the literature [13, 16, 18, 19, 21, 22]. The plasma membrane appears to be a heterogeneous structure containing different lipid domains as well as a nonhomogeneous distribution of membrane-bound proteins [30–32]. The linear correlation between the effects of ethanol and butanol on fluorescence anisotropy and adenylate cyclase activity could be merely fortuitous, or could indicate that the enzyme is sensitive to changes in bulk membrane order. The quantitative difference between the effects of the two alcohols may be due to differential interactions with the lipid microenvironment in the vicinity of the enzyme. Alterations in the bulk order of the membrane as a whole may not translate into parallel changes in the microenvironment of specific enzymes such as adenylate cyclase, and the present study emphasizes the hazard in interpreting changes in bulk membrane order as being causally related to changes in the activities of specific enzymes.

Although the ability of ethanol to increase adenylate cyclase activity is not entirely due to a decrease in bulk membrane order, alcohol-induced activation of the enzyme does appear to involve an interaction of ethanol with a hydrophobic region(s) of the membrane. Thus, in membranes prepared from L6 cells, butanol, which is more lipid-soluble than ethanol, was more potent in augmenting isoproterenol-stimulated adenylate cyclase activity than was ethanol. These results are in agreement with the previously reported order of potency for short-chain alcohols for increasing adenylate cyclase activity in a variety of tissues [5, 7, 10, 11]. At the present time, however, it is not known whether ethanol is exerting a direct effect on a hydrophobic region of one or more of the components of the adenylate cyclase system or on

the lipid bilayer in the microenvironment of the enzyme.

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