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Basal and adenosine receptor-stimulated levels of cAMP are reduced in lymphocytes from alcoholic patients

(alcoholism/adenylate cyclase/ethanol)

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ABSTRACT **Alcoholism causes serious neurologic disease that may be due, in part, to the ability of ethanol to interact with neural cell membranes and change neuronal function. Adenosine receptors are membrane-bound proteins that appear to mediate some of the effects of ethanol in the brain. Human lymphocytes also have adenosine receptors, and their activation causes increases in cAMP levels. To test the hypothesis that basal and adenosine receptor-stimulated cAMP levels in lymphocytes might be abnormal in alcoholism, we studied lymphocytes from 10 alcoholic subjects, 10 age- and sex-matched normal individuals, and 10 patients with nonalcoholic liver disease. Basal and adenosine receptor-stimulated cAMP levels were reduced 75% in lymphocytes from alcoholic subjects. Also, there was a 76% reduction in ethanol stimulation of cAMP accumulation in lymphocytes from alcoholics. Similar results were demonstrable in isolated T cells. Unlike other laboratory tests examined, these measurements appeared to distinguish alcoholics from normal subjects and from patients with nonalcoholic liver disease. Reduced basal and adenosine receptor-stimulated levels of cAMP in lymphocytes from alcoholics may reflect a change in cell membranes due either to chronic alcohol abuse or to a genetic predisposition unique to alcoholic subjects.**

Despite the widespread incidence of alcoholism, the molecular events accounting for intoxication, tolerance, and physical dependence after alcohol abuse are poorly understood. Ethanol is believed to intercalate into cell membranes, producing acute and adaptive changes in membrane fluidity (1, 2) or membrane constituents (3, 4). Adenosine receptors are membrane-bound proteins that appear to mediate some of the effects of ethanol in the central nervous system (5). We recently reported (6) that ethanol acutely increases adenosine receptor-stimulated cAMP levels in a clonal neural cell line (neuroblastoma–glioma hybrid NG108-15) and that with time these cells adapt to the presence of ethanol, showing a reduction in adenosine receptor-stimulated cAMP levels. After chronic exposure to ethanol, the NG108-15 cells require ethanol to achieve normal levels of adenosine receptor-stimulated cAMP, which may indicate a form of cellular "dependence." This process is completely reversible upon ethanol withdrawal.

These observations suggest that intact single cells adapt to the presence of ethanol. Moreover, depressed adenosine receptor-stimulated cAMP levels in cells might be a biochemical change of pathophysiologic significance in chronic alcoholism. Since human lymphocytes have the same A_2 adenosine receptors as the neuroblastoma cells (7), we could test directly whether alcoholics have altered cAMP levels. We undertook a controlled study of basal and adenosine receptor-stimulated cAMP levels in lymphocytes of chronic alcoholics, normal subjects, and patients with nonalcoholic liver disease. Mixed lymphocytes and T cells from chronic alcoholics showed a striking reduction in basal and adenosine receptor-stimulated cAMP levels. Moreover, these lymphocytes showed striking resistance to ethanol stimulation of adenosine receptor-dependent cAMP levels.

MATERIALS AND METHODS

Description of Patients. Ten volunteer, actively drinking alcoholic patients from the Ernest Gallo Clinic and Research Center (an evaluation and treatment unit for alcoholic neurologic disorders at San Francisco General Hospital) were matched for age and sex with 10 normal individuals (9 men and 1 woman) from a local Mormon church group, a local high school alumni association, and the hospital staff. Each alcoholic was also age-matched with a nonalcoholic outpatient with liver disease from the Gastroenterology Clinic of the University of California, San Francisco. The liver disease group was not sex-matched (5 men and 5 women). Estimates of lifetime ethanol consumption were based on medical chart review and a patient questionnaire from which ethanol consumption was derived by the formula: lifetime ethanol consumption (kg) = [(grams of ethanol per serving) × (servings per day) × (days per month drinking) × (12) × (years of drinking)] ÷ 1000. Blood ethanol levels were measured at the time blood was drawn for the lymphocyte cAMP studies.

Materials. Heparinized Vacutainers were purchased from Becton Dickinson. Histopaque-1077 and RIA-grade bovine serum albumin were obtained from Sigma. N^6-(*R*-phenylisopropyl)adenosine (Ph*i*PrAdo) and fatty acid-free bovine serum albumin were obtained from Boehringer Mannheim. Ro 20-1724 was a gift from Hoffmann–La Roche. Rabbit antiserum raised against cAMP conjugated to bovine serum albumin was obtained from Miles. ^{125}I-labeled cAMP was provided by Chemicon (Los Angeles). Sheep whole blood in Alsever's solution was purchased from Hana Media (Berkeley, CA). Cell-culture-grade 100 × 20-mm plastic Petri dishes and 12 × 75-mm polypropylene tubes were purchased from Falcon.

Cell Preparation. Human lymphocytes were prepared by established procedures (8). Peripheral blood was collected in heparinized glass tubes (143 USP units/10 ml). Platelet contamination was reduced in the cell preparation by centrifuging 30-ml aliquots of blood for 20 min at 100 × *g* and removing the upper, platelet-rich layer. The remaining blood was diluted 1:3 with calcium/magnesium-free Hanks' balanced salt solution (HBSS) containing 25 mM Hepes (pH 7.2). Ten milliliters of Ficoll (density = 1.077 g/cm^3) were layered under 40 ml of the diluted blood in a 50-ml centrifuge

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Abbreviation: Ph*i*PrAdo, N^6-(*R*-phenylisopropyl)adenosine.
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tube. After a 25-min centrifugation at 400 × *g* at room temperature, the mononuclear cells, including B and T lymphocytes and monocytes, were removed from the Ficoll/plasma interface with a glass pipette. The cells were washed twice in HBSS and suspended at a concentration of 10^6 cells per ml in Dulbecco's phosphate-buffered saline (DPBS) containing 0.2% glucose and 25 mM Hepes (pH 7.2). Cell viability, assessed by trypan blue exclusion, averaged 95%. This mixed-lymphocyte preparation was used in all studies except for experiments with T cells described in Fig. 2.

Isolation of T and B Cells. T cells were separated by erythrocyte (E)-rosette formation, using a modification of the method of Kasakura *et al.* (9). In order to isolate T cells, the heterogeneous lymphocyte preparation was resuspended after the final wash in HBSS to a concentration of 5–10 × 10^6 cells per ml in sheep erythrocyte-absorbed DPBS containing 0.2% glucose and 5% bovine serum albumin (fatty acid-free). The DPBS solution was also used to prepare a 3% suspension of washed, packed sheep erythrocytes. Eight milliliters of both cell suspensions were mixed in a plastic centrifuge tube and kept at room temperature for 10 min. Ten milliliters of Ficoll was then layered under the mixture and the tubes were centrifuged for 30 min at 400 × *g* at room temperature. Rosettes (E^+) of human T lymphocytes and sheep erythrocytes formed a pellet at the bottom of the tube, while nonrosetting (E^-) B cells and monocytes collected at the Ficoll interface. Erythrocytes in the E^+ pellet were lysed with lysing reagent (Ortho Diagnostics) and the remaining T cells were washed twice with calcium/magnesium-free DPBS and resuspended at 10^6 cells per ml in RPMI-1640 medium supplemented with 2% bovine serum albumin, 2 mM L-glutamine, antibiotics (50 units of penicillin per ml and 50 μg of streptomycin per ml), and 25 mM Hepes (pH 7.2). T cells from alcoholics and control subjects were carried through all preparations and analytical procedures simultaneously.

B cells and monocytes were removed from the Ficoll interface, washed twice with calcium/magnesium-free DPBS, and resuspended at a concentration of 10^6 cells per ml in supplemented RPMI-1640 without Hepes. This cell suspension was then poured into plastic Petri dishes (10 ml per dish) and held for 120 min at 37°C in a humidified 7% CO_2 incubator. Monocytes adhered to the dish, and B cells in the suspension were removed and washed twice with DPBS, resuspended at 10^6 cells per ml in supplemented RPMI-1640, and kept at 4°C.

Both B- and T-cell suspensions were incubated with 7.3 mM L-leucine methyl ester for 45 min at room temperature to eliminate contaminating monocytes prior to use (10). The reaction was stopped by the addition of 1 ml of fetal bovine serum. The cells were washed twice with DPBS and suspended at a concentration of 10^6 cells per ml in DPBS containing 0.2% glucose and 25 mM Hepes (pH 7.2).

Assay for Basal and Stimulated cAMP in Cells. Cells (5 × 10^5 in 0.5 ml) were preincubated in triplicate in 12 × 75-mm polypropylene tubes for 5 min with 90 μM Ro 20-1724, a phosphodiesterase inhibitor, and then incubated for 60 min with or without 80 μM Ph*i*PrAdo, an adenosine receptor agonist, or 80 mM EtOH in a final volume of 0.6 ml. The incubation was stopped by adding 50 μl of 2% Nonidet P-40 in 1 M HCl and extracting the cells on ice for 10 min. cAMP levels were determined in a 700 × *g* supernatant by radioimmunoassay (11). cAMP concentrations were determined without investigator bias by using an automated nonlinear regression analysis procedure on a Beckman DP5500 gamma counter. Protein was measured as described by Lowry *et al.* (12), using bovine serum albumin as standard. Standard conditions for cAMP determination were first established using 31 preparations of lymphocytes from normal volunteers.

RESULTS

Each of the alcoholic patients had normal weight for height, based on the Metropolitan Life Insurance height/weight table, and each individual was considered to be well-nourished by an experienced clinician (W.E.), who examined all of the patients in this study. Five alcoholics had normal neurologic examinations. The other five alcoholic patients had mild memory deficits (3), mild peripheral neuropathy (2), and mild cerebellar gait ataxia (2). Diagnoses in the liver disease group included hemochromatosis (2), chronic active hepatitis (2), postnecrotic cirrhosis, metastatic carcinoma (breast), viral hepatitis, "autoimmune" disease with hepatitis, idiopathic elevation of liver enzymes, and cryptogenic cirrhosis. The mean lifetime consumption of ethanol in the alcoholic group was 13 times greater than in the control group and 43 times greater than in the liver disease group (Table 1). Two alcoholics in the study had blood ethanol levels of 1 and 2.5 mg/ml, respectively, while the remaining patients had no detectable blood ethanol. Table 1 summarizes the results of clinical laboratory measurements for the alcoholics, patients with nonalcoholic liver disease, and control subjects. There was no evidence of malnutrition in the alcoholic subjects. Their hemoglobin levels and mean corpuscular volumes were normal, indicating adequate iron and folic acid ingestion. Transketolase activity was normal, suggesting an adequate thiamine-containing diet, and such markers for malnutrition as serum albumin and lymphocyte counts were also normal. Alcoholics and patients with liver disease exhibited abnormalities of some liver enzymes, but there were no statistically significant differences between the alcoholic and matched liver disease groups. Laboratory values in alcoholic patients were similar to normal controls except for significantly higher serum glutamic-oxaloacetic transaminase and globulin.

Ph*i*PrAdo is an adenosine receptor agonist that is not readily transported into cells (13). Consistent with earlier reports of the lymphocyte A_2 receptor (7, 14), cAMP stimulation by Ph*i*PrAdo was blocked by xanthines (data not shown). Under standard conditions of assay, unstimulated (basal) and Ph*i*PrAdo-stimulated levels of cAMP in normal human lymphocyte preparations (n = 31) were 8.97 ± 0.88 (SEM) and 14.85 ± 1.37 pmol per 10^6 cells, respectively. Ph*i*PrAdo-stimulated cAMP accumulation varied as a linear function of cell number, and ethanol added *in vitro* further increased Ph*i*PrAdo-stimulated cAMP levels in a concentration-dependent manner without changing basal cAMP levels (data not shown). There was no correlation of basal or stimulated cAMP levels with age or sex, but day-to-day variation was noted in some subjects.

Alcoholics showed highly significant depression of basal and Ph*i*PrAdo-stimulated cAMP levels in intact lymphocytes when compared to normal subjects (Fig. 1) or patients with liver disease. P values were calculated by Wilcoxon rank sum test comparing controls to alcoholics and alcoholics to patients with liver disease. Mean basal cAMP levels (pmol per 10^6 cells) in controls, alcoholics, and patients with liver disease were 9.55 ± 1.65 (SEM), 2.30 ± 0.34 (P = 0.0004), and 8.33 ± 1.29 (P = 0.0005), respectively. Mean Ph*i*PrAdo-stimulated cAMP levels were 15.81 ± 2.52, 3.72 ± 0.53 (P = 0.0002), and 14.04 ± 1.93 (P = 0.0005), respectively. The difference between alcoholics and controls was also striking when the effect of 80 mM ethanol on Ph*i*PrAdo-stimulated cAMP levels was compared (Fig. 1). In addition, the percent response to ethanol was decreased markedly in lymphocytes from alcoholics compared to cells from normal subjects. Ethanol increased Ph*i*PrAdo-stimulated cAMP levels by 17.0

Table 1. Clinical values (mean ± SEM) for control subjects, alcoholics, and patients with nonalcoholic, noninfectious liver disease

Parameter	Value: Controls	Value: Alcoholics	Value: Liver disease patients
Age, years	49.6 ± 3.6	48.6 ± 3.6	45.3 ± 4.0
Lifetime ethanol, kg	145.4 ± 76.9	1914.0 ± 319.0*	44.9 ± 23.1†
Blood analysis			
Leukocyte count, no. × 10^{-3} per μl	5.9 ± 0.5	6.6 ± 0.6	6.8 ± 0.4
Lymphocyte count, no. × 10^{-3} per μl	1.5 ± 0.1	2.3 ± 0.5	2.1 ± 0.4
Hemoglobin, g/dl	14.5 ± 0.5	14.5 ± 0.6	14.2 ± 0.4
Mean erythrocyte volume, μm^3	90.0 ± 3.0	89.6 ± 1.5	92.1 ± 2.1
Glucose, mg/dl	71.1 ± 12.2	133.2 ± 49.4	102.0 ± 21.1
Urea nitrogen, mg/dl	14.1 ± 1.0	9.6 ± 1.7	12.3 ± 0.9
Creatinine, mg/dl	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
Albumin, g/dl	5.1 ± 0.6	4.3 ± 0.1	4.0 ± 0.3
Globulin, g/dl	2.4 ± 0.1	3.2 ± 0.3‡	3.7 ± 0.8
Uric acid, mg/dl	6.2 ± 0.4	5.6 ± 0.6	6.2 ± 0.8
Cholesterol, mg/dl	219.4 ± 11.1	238.3 ± 30.2	208.3 ± 21.8
Triacylglycerol, mg/dl	158.1 ± 29.2	152.0 ± 39.2	142.5 ± 24.7
Bilirubin, mg/dl	0.6 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
Alkaline phosphatase, units/liter	74.0 ± 10.1	79.3 ± 17.7	123.4 ± 25.7
Lactate dehydrogenase, units/liter	220.1 ± 21.4	174.4 ± 13.6	216.0 ± 27.6
Serum glutamic-pyruvic transaminase (SGPT), units/liter	34.4 ± 7.0	51.6 ± 18.7	119.6 ± 38.6
Serum glutamic-oxaloacetic transaminase (SGOT), units/liter	23.1 ± 1.9	58.7 ± 16.3§	102.0 ± 25.4
SGPT/SGOT ratio	1.43 ± 0.17	0.90 ± 0.15	1.31 ± 0.26
γ-Glutamyltransferase, units/liter	19.9 ± 9.6	76.6 ± 22.5	68.8 ± 49.3
Transketolase, units/g of Hb	0.99 ± 0.05	0.89 ± 0.07	0.91 ± 0.06

*$P = 0.001$ compared to control group. (P values were calculated by Wilcoxon rank sum test comparing controls to alcoholics and alcoholics to patients with liver disease.)
†$P = 0.001$ compared to alcoholic group.
‡$P = 0.007$ compared to control group.
§$P = 0.04$ compared to control group.

± 3.8% (SEM) in lymphocytes from alcoholics compared to 71.0 ± 9.6% in normal individuals ($P = 0.002$). The mean cAMP level in Ph*i*PrAdo-stimulated lymphocytes from alcoholics after addition of ethanol was 4.33 ± 0.96 (SEM) pmol per 10^6 cells, while the value for normal controls was 28.0 ± 5.2.

Most of the adenosine receptors in lymphocytes appear to be associated with the T-cell fraction (15), although this is controversial (16). It is possible that a reduction in the percentage of circulating T cells in alcoholics (17, 18) might account for the reduced cAMP levels in alcoholics. To confirm that these reduced levels of stimulated cAMP in cells of alcoholics were not due to changes in the lymphocyte population, T cells from unmatched normal and alcoholic subjects were isolated and assayed separately (9, 10). Fig. 2 shows that there was also a marked reduction in basal ($P < 0.02$) and Ph*i*PrAdo-stimulated cAMP levels ($P < 0.03$) in T cells recovered from alcoholic patients compared to controls. We found no significant effect of Ph*i*PrAdo on cAMP accumulation in B cells from alcoholics or controls (data not shown). Basal levels of cAMP were similar in B and T cells

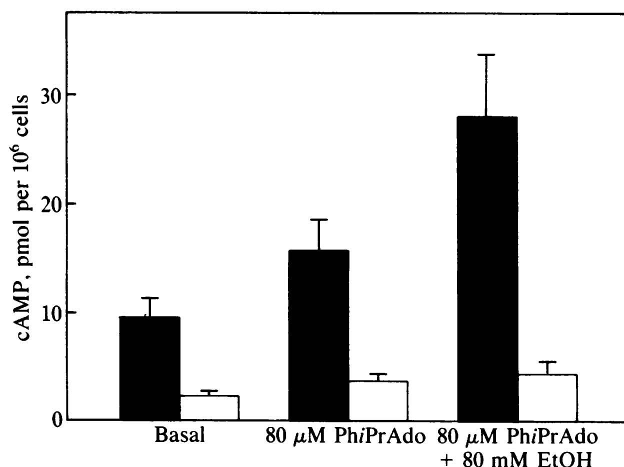

FIG. 1. Basal and stimulated cAMP levels in lymphocytes from alcoholics (open bars) and control subjects (solid bars). Ph*i*PrAdo is an adenosine receptor agonist. Each bar represents the mean ± SEM ($n = 10$ for basal and Ph*i*PrAdo; $n = 9$ for Ph*i*PrAdo plus ethanol).

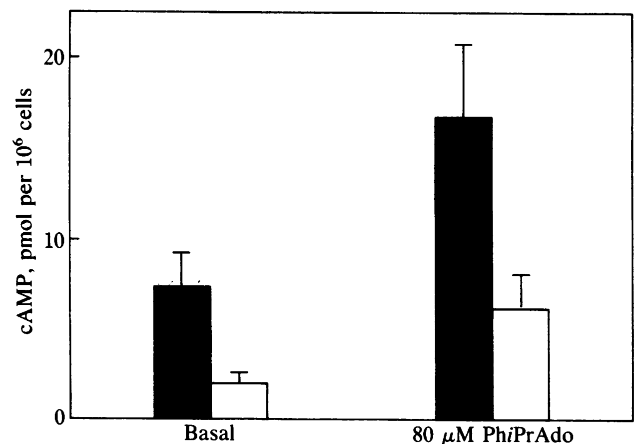

FIG. 2. Basal and Ph*i*PrAdo-stimulated cAMP levels in T cells from alcoholics (open bars) and control subjects (solid bars). Each bar represents the mean ± SEM ($n = 6$).

from each individual alcoholic or control subject studied (data not shown).

DISCUSSION

Adenosine is an inhibitory neuromodulator (19) that may play a role in mediating the effects of ethanol in the central nervous system (5, 20). We have found (6) that adenosine receptor-stimulated cAMP levels in NG108-15 neural cells are increased by ethanol added acutely and that these levels become depressed after chronic exposure to ethanol. This adaptive response appears to compensate for the acute activating effect of ethanol and may represent a form of cellular tolerance. Adenosine also interacts with receptors on human lymphocytes to stimulate the accumulation of cAMP (7, 14, 21), raising the possibility that this system might be affected by chronic ethanol abuse in humans. Indeed, lymphocytes from chronic alcoholics had about a 75% reduction in basal and adenosine receptor-stimulated levels of cAMP when compared to lymphocytes from normal individuals or patients with nonalcoholic liver disease. The reduction in basal cAMP levels in lymphocytes from alcoholic patients cannot readily be explained by changes in the percentage of circulating T cells (17) or B cells, since total lymphocyte counts were normal and isolated B cells and T cells had similar basal cAMP levels in each subject studied.

Our results suggest that lymphocytes from alcoholics are distinguishable from the lymphocytes of nonalcoholic subjects. Cells from alcoholics exhibited reduced basal cAMP levels, reduced Ph*i*PrAdo-stimulated cAMP levels, and increased resistance to ethanol stimulation of adenosine receptor-dependent cAMP accumulation. Membrane changes induced by alcoholism might be expected to increase resistance to ethanol added acutely (1–3). We found that lymphocytes from alcoholics were strikingly resistant to the acute stimulating effect of ethanol added *in vitro*. There was a 76% reduction in the percent ethanol stimulation of Ph*i*PrAdo-dependent cAMP accumulation in lymphocytes from alcoholics compared to lymphocytes from controls. Our measurements of basal and receptor-stimulated cAMP levels in lymphocytes may be valuable as a sensitive test for distinguishing between alcoholic and nonalcoholic human populations. The reduced levels of cAMP in lymphocytes from alcoholics may reflect an acquired membrane abnormality caused by chronic alcohol abuse. On the other hand, our findings could be related to a genetically determined difference in the membrane response of cells from alcoholics (22). Studies of ethanol interaction with lymphocytes from alcoholics and controls maintained over long periods of time *in vitro* may help to explain the molecular basis of this functional membrane defect and might identify individuals at risk to develop alcoholism.

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