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Adenylyl Cyclase Signal Transduction and Alcohol-Induced Sedation

JANICE C. FROEHLICH* AND GARY S. WAND†

*Departments of Medicine and Physiology/Biophysics, Indiana University School of Medicine, Indianapolis, IN 46202
†Departments of Medicine and Psychiatry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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FROEHLICH, J. C. AND G. S. WAND. *Adenylyl cyclase signal transduction and alcohol-induced sedation*. PHARMACOL BIOCHEM BEHAV **58**(4) 1021–1030, 1997.—This study examined adenylyl cyclase (AC) signal transduction in alcohol-sensitive brain regions of rats selectively bred for high (HAD) and low (LAD) alcohol drinking and correlated these findings with differences in sensitivity and tolerance to alcohol-induced sedation found within these lines. LAD rats were more sensitive to the sedative effects of alcohol than were HAD rats as evidenced by a shorter latency to lose the righting response (RR) after a single alcohol challenge. When time to recover the RR was compared after each of two alcohol challenges, HAD rats recovered the RR more rapidly following the second challenge compared to the first, indicating that the HAD rats rapidly developed tolerance to the sedative effects of alcohol. Tolerance did not develop in rats of the LAD line. Two months after completion of behavioral testing, adenylyl cyclase (AC) signal transduction was examined in alcohol-sensitive brain regions of rats from both lines. Immunoblot analyses indicated that LAD rats had greater $G_s\alpha$ expression in the frontal cortex (FC) and hippocampus (HIP) compared to HAD rats. Rats with the highest HIP and FC $G_s\alpha$ levels were more rapidly affected by the sedative properties of alcohol than were rats with lower $G_s\alpha$ levels. G protein expression and AC activity in the FC, HIP, cerebellum (CERE), and nucleus accumbens (ACB) were also correlated with sensitivity to the sedative properties of alcohol and with the rapid development of tolerance to this alcohol effect. The results suggest that sensitivity and tolerance to alcohol-induced sedation may be mediated in part through AC signal transduction. © 1997 Elsevier Science Inc.

Genetic selection Alcohol drinking Alcohol sensitivity Alcohol tolerance Adenylyl cyclase cAMP G proteins

A GENETIC propensity toward high alcohol drinking is very likely determined by multiple genes that regulate a number of predisposing neurobiological traits. When various rodent lines and strains are examined, the most generalizable and robust traits that have been found to be associated with high alcohol drinking are low initial sensitivity to alcohol and the rapid development of alcohol tolerance (9,13,15,16,20,36,42). Initial sensitivity to alcohol might be expected to contribute to high alcohol intake because organisms that are initially less sensitive (i.e., more resistant) have to consume more alcohol to produce the same magnitude of drug effect as that produced by a smaller amount of alcohol in sensitive organisms. We have previously demonstrated that rats selectively bred for high alcohol drinking (the alcohol preferring or P line) are less sensitive to the sedative and motor-impairing effects of alcohol than are those bred for low alcohol drinking (10,15,18,25).

Alcohol tolerance is a central diagnostic criterium of alcoholism and recent evidence suggests that "tolerance-proneness" may be an important risk factor for heavy alcohol drinking and for the subsequent development of alcoholism. Alcohol tolerance may serve to increase alcohol intake in either of two ways. Tolerance developed to the aversive effects of alcohol might be expected to increase intake by attenuating the negative physiological consequences that would otherwise serve to limit subsequent intake (2). Tolerance developed to the reinforcing effects of alcohol would require an increase in intake to maintain the same level of drug effect (41). It has previously been demonstrated that P rats rapidly develop tolerance to many of the behavioral and physiological effects of alcohol such as motor impairment, hypothermia, and sedation (11,15, 19,37,38,45) while rats selectively bred for low alcohol drinking (the alcohol nonpreferring or NP line) do not.

Requests for reprints should be addressed to Gary S. Wand, M.D., The Johns Hopkins University School of Medicine, Ross Research Building, Room 850, 720 Rutland Avenue, Baltimore, MD 21205.

When using selectively bred rodent lines to identify traits that are associated with, and may be causally related to, a given phenotype it is important to demonstrate that the relationship between the phenotype and the associated trait exists in more than one line selected for the same phenotype (4). The appearance of the associated trait in two or more lines powerfully decreases the likelihood that the observed relationship is fortuitous. Recognizing the importance of this approach, the first goal of the present study was to determine whether the association between sensitivity and tolerance to the sedative properties of alcohol seen in P and NP lines can be generalized to HAD and LAD lines.

The neuronal circuitry and brain pathways that underlie initial sensitivity to the sedative properties of alcohol and the rapid development of tolerance to this effect are not clear. We posit that the adenylyl cyclase (AC) signal transduction system is important in mediating the neuronal changes underlying initial sensitivity to alcohol and the rapid development of alcohol tolerance. This view is supported by several lines of evidence. First, the membrane AC signal transduction pathway is altered by acute and chronic alcohol treatment (12). In general, a single alcohol challenge potentiates agonist-stimulated AC activity that may be a potential mechanism for regulating initial sensitivity to alcohol. In contrast, two or more exposures to alcohol are associated with the development of desensitization within the AC system, which is a potential mechanism underlying alcohol tolerance. Second, alcoholics have abnormal AC signal transduction and these abnormalities may, in part, be genetically determined (6,40,50). We postulate that genetic differences in the AC signal transduction system allow for differential amplification of receptor-stimulated cAMP accumulation following alcohol exposure and that the resulting differences in cAMP levels, in turn, allow for differences in the magnitude of certain physiological and behavioral responses to alcohol. Viewed in this way, alcohol-induced changes in AC activity may represent the "source" of initial sensitivity to alcohol and alcohol tolerance, both of which are then expressed as physiological and behavioral changes. One approach to testing this hypothesis is to determine whether a correlation exists between membrane AC activity (e.g., potential energy to drive a physiological or behavioral response) and the magnitude of the response to an alcohol challenge (e.g., realized or expressed energy).

In the present study, we compared sensitivity and tolerance to the sedative properties of alcohol in rats selectively bred for alcohol preference (HAD line) and nonpreference (LAD line). We also examined various elements of the AC signal transduction system in alcohol-sensitive brain regions that are likely to be involved in mediating alcohol-induced sedation and correlated these findings with the differences in sensitivity and tolerance found within these selected lines.

METHOD

Subjects

Selective breeding for high and low alcohol preference was used to derive the P and HAD rat lines that consume large amounts of alcohol and the NP and LAD lines that drink very little alcohol. Within each generation, rats were selected for breeding based on their intake of alcohol during 4 weeks of free choice between a 10% (v/v) alcohol solution and water with food freely available (17,18,24,25,27). Rats selected for breeding in the high alcohol drinking lines (P and HAD) are those that consume in excess of 5.0 g alcohol/kg b.wt./day and demonstrate a 2:1 preference ratio of alcohol to water. Rats selected for breeding in the low alcohol drinking lines (NP and LAD) are those that consume less than 0.5 g alcohol/kg b.wt./day and demonstrate a preference ratio that does not exceed 0.2:1.

In the present study, male rats weighing 337–429 g, obtained from the 18th generation of the HAD and LAD lines, served as subjects. Rats were housed individually in standard steel laboratory cages in a quiet room under controlled temperature and lighting conditions (lights on 0700–1900 h), with water and standard laboratory food pellets (Purina, #5001) freely available.

Alcohol preference testing. All subjects were tested for alcohol preference using the procedures and criteria which are routinely used in the derivation of the P, NP, HAD, and LAD lines. Briefly, alcohol and water intake was calculated daily during 4 weeks of a free choice between a 10% (v/v) alcohol solution and water with food freely available. Fluids were presented in calibrated glass Richter tubes that were read to the nearest 0.5 ml. The position of the Richter tubes was alternated daily to minimize the effect of any possible positional preference. Average daily alcohol intake was 8.35 g/kg b.wt./day for rats from the HAD line and 0.19 g/kg b.wt./day for rats from the LAD line. Access to alcohol was terminated following completion of alcohol preference testing and subjects were maintained without alcohol but with ad lib access to food and water for 4 months prior to initiation of behavioral testing for alcohol sensitivity and tolerance.

Loss and Recovery of the Righting Response as a Measure of Sensitivity and Tolerance to Alcohol-Induced Sedation

Sensitivity to alcohol is generally measured by examining one or more features of the response to a single alcohol challenge (14,39,45). The time required to lose a function following a single alcohol challenge is a particularly useful measure of sensitivity because it is not confounded by the possible development of within-session tolerance that can occur with other measures (30,45). In the present study time to loss of the righting response after a single alcohol challenge was used to index sensitivity to the sedative effects of alcohol.

Alcohol tolerance is defined as a decrease in the magnitude of response to a second or subsequent alcohol challenge (41). One method for assessing alcohol tolerance is to administer two alcohol challenges and to compare the magnitude of a given response after each. Alcohol tolerance is defined as a faster behavioral recovery following the second alcohol challenge compared with the first. In the present study alcohol-induced loss of the righting response (RR) and recovery of RR were measured following each of two alcohol injections that were administered 1 day apart as previously described (15). Alcohol was injected IP in a dose of 3.0 g alcohol/31.5 ml/kg b.wt. between 0800 and 1100 h. The concentration of the alcohol solution did not exceed 12% (v/v in saline) to minimize concentration-induced differences in alcohol absorption rate and tissue irritation at the site of injection (1,23).

Latency to lose the RR was used to index sensitivity to the sedative effects of alcohol, with a shorter latency reflecting increased sensitivity. The apparatus used to measure the RR was a V-shaped trough which was bent at a 90° angle and lined with sandpaper. The troughs were contained in a temperature controlled chamber that served to reduce sound disturbances. To determine loss of RR, each rat was placed on its back in the trough once every 30 s after the alcohol injection until it was unable to right itself within a 30-s interval. Latency to lose RR was defined as the time between the onset of injection and the beginning of the 30-s interval during which the rat was

unable to right itself. The rat was then left undisturbed on its back until it turned over so that its forelegs were under its body and not visible from directly above. At this time righting trials were initiated. A righting trial was begun by placing the rat on its back. Righting was defined as the rat achieving the orientation in which at least three feet were under its body and not visible from above. If the rat righted itself within 15 s, a second righting trial was immediately initiated. Recovery of the RR was defined as the ability of the rat to right itself in two consecutive 15-s trials. If the rat was unable to right itself in the first 15-s trial, it was left undisturbed until it turned over again, at which time righting trials were reinitiated. Sleep time was defined as the time interval between loss and recovery of RR. Tolerance was defined as a significantly faster recovery of the RR following the second alcohol injection compared with the first.

Brain dissection. Following completion of behavioral testing for alcohol sensitivity and tolerance, all subjects were maintained with ad lib access to food and water for 2 months prior to decapitation and brain dissection. The following brain areas were dissected according to the coordinates of Paxinos and Watson (33) using bregma as the reference point: cerebellum, frontal cortex, hippocampus, hypothalamus, nucleus accumbens, ventral tegmental area, lateral septum, anterior, and posterior striatum. The pituitary was removed from the sella turcica with curved forceps and the anterior and neurointermediate lobes separated.

Membrane preparation. Membranes were prepared as previously described (49). Tissue homogenates were spun at 500 × *g* for 5 min at 4°C to remove the nuclear pellet. The supernatant was then spun at 20,000 × *g* for 20 min at 4°C. The pellet was washed twice in wash buffer (lysis buffer without sucrose). The final membrane pellet was diluted with wash buffer and stored at −70°C. Protein concentration was determined by bicinchronic acid reaction with BSA standard.

Adenylyl cyclase activity. Membranes were prepared as previously described (51). AC activity of membrane preparations was determined in triplicate using a modification of the method by Salomon (35) as previously described (49). Aliquots (10 μg) of membrane protein were assayed at 30°C for 30 min with no further additions (basal) or in the presence of 10 μM GTPγS (guanosine 5;-0-(3-thotriposphate) or 1 μM Forskolin. The reaction was terminated by adding 100 μl of 50 mM Hepes (pH 7.5, 2 mM ATP, 0.5 mM cAMP, 2% SDS, [^{3}H] (15 nc_i) to each tube and heating to 100°C for 3 min. Cyclic AMP was isolated by chromatography (35). Recovery averaged 85%.

Immunoblot Analysis

For immunoblot analysis (49), membrane proteins (50 μg) were fractionated by electrophoresis through 10% SDS–polyacrylamide gels (10% acrylamide, 0.13% phenylpiperazine). Proteins were electrophoretically transferred to polyvinylidine difluoride filters using a transfer bath containing 10% methanol, 0.01 mM 3-(cyclohexlamino)-1-Propanesulfonic acid, pH 11.0). Filters were incubated for 2 h at room temperature in 50 mM Tris, 138 mM NaCl, 2 mM $MgCl^2$, pH 7.4 T(TBS) containing 3% BSA, 0.1% Tween-20, 0.02% NaN_3 and washed twice for 5 to 10 min with TBS containing 0.2% SDS, 2% Nonidet P-40. Filters were incubated overnight at room temperature with specific primary antibodies in TBS containing 1% BSA, 0.05% Tween-20, 0.02% NaN_3, and 2% Nonidet P-40. Filters were washed twice for 30 min in wash buffer (TBS, 0.2% SDS, 2% Nonidet P-40) and incubated for 2 h with ^{125}I-protein A (0.5 μCi/ml) in 1% BSA, 0.5% Tween-20 0.02% NaN_3, and 2% Nonidet P-40. Filters were washed twice for 30 min and then rinsed twice for 5 to 10 min in wash buffer and autoradiographed. The 52 kDa and 45 kDa forms of $G_s\alpha$ were identified employing antisera 1805 (NEN); $G_i2\alpha$ was identified employing antisera A7 (NEN). Autoradiographic image intensities were determined by two-dimensional densitometry using the Molecular Dynamics personal densitometer system.

Control for gel loading and transfer. Immediately after protein transfer to PDVF membranes and just prior to blocking, membranes were stained with coumassie blue to insure equal loading and transfer. In rare instances where this was not the case, the procedure was terminated at that step and another gel was prepared for repetition of the immunoblot.

Quantification of data. Twenty samples were run per gel. Twenty lanes per gel allowed for the processing of all HAD samples ($n = 8$) and all LAD samples ($n = 8$) from a given brain region on the same gel. Also included on the gel was a standard membrane preparation composed of all 16 samples. The standard was run in quadruplicate. G protein measurements for a specific rat area were then expressed relative to the density of the standard sample. This process was repeated three times (e.g., each membrane sample is run on three separate gels) to get an average G protein density measurement. The coefficient of variance (CV) was usually less than 15%. When the CV was greater than 15%, samples were rerun until this value was achieved. The amount of $G_s\alpha$ expression determined for each tissue is reported as the sum of the 52 kDa and 45 kDa forms of $G_s\alpha$.

Statistical Analysis

Latency to lose the RR (alcohol sensitivity) was compared in rats of the HAD vs. LAD lines after a single alcohol challenge using simple factorial ANOVAs. Latency to lose the RR following each of two alcohol challenges was compared within each line using a 2 × 2 repeated-measures ANOVA. A difference score was also obtained for each rat by subtracting the time to lose the RR following the first alcohol challenge from the time to lose the RR following the second challenge. Student's *t*-tests were used to determine if the mean difference score for each line was significantly different from zero.

Sleep time, or time to regain RR, was defined as the time to loss of RR minus time of recovery of RR. Sleep time was compared in rats of the HAD vs. LAD line after a single alcohol challenge using simple factorial ANOVAs. Sleep time following each of two alcohol challenges was compared within each line using a 2 × 2 repeated-measures ANOVA. A difference score was also obtained for each rat by subtracting the duration of alcohol-induced sedation (sleep time) following the first alcohol challenge from sleep time following the second challenge. Student's *t*-tests were used to determine if the mean difference score for each line was significantly less than zero (indicating tolerance) or greater than zero (indicating sensitization).

Correlations between the following variables were analyzed: $G_s\alpha$, G_i2_α, adenylyl cyclase activity, nine brain regions, behavioral tolerance, and initial sensitivity to ethanol. Pearson product moment correlation coefficients were calculated using SAS version 6.09. The accepted significance level for all statistical tests was $p < 0.05$.

RESULTS

Alcohol Sensitivity

Rats of the LAD line exhibited a shorter latency to lose the RR than did rats of the HAD line ($F = 12.508$, $p = 0.003$) after a

single alcohol challenge (challenge 1), which indicates that rats of the LAD line are more sensitive to the sedative effects of alcohol (Fig. 1A). When latency to lose the RR was compared after each of two alcohol challenges, HAD rats had a shorter latency to lose the RR after the second challenge compared with the first ($F = 6.961, p = 0.034$), while LAD rats exhibited similar latencies (Fig. 1A). This finding was reflected in a significant ($p < 0.05$) difference score in rats of the HAD line (Fig. 1B).

Alcohol Tolerance

Rats of the HAD and LAD lines did not differ in duration of alcohol-induced sedation (sleep time) following a single alcohol challenge (Fig. 2A). However, HAD rats recovered the RR more rapidly following a second alcohol challenge compared with the first ($F = 5.069, p = 0.059$), indicating that the HAD rats developed tolerance to the sedative effects of alcohol (Fig. 2A). This finding was reflected in a significant ($p < 0.05$) difference score in rats of the HAD line (Fig. 2B). By contrast, duration of alcohol-induced sedation (sleep time) was similar after each of the two alcohol challenges in rats of the LAD line (Fig. 2A).

G Protein Expression and Adenylyl Cyclase Activity

Two months following completion of behavioral testing subjects were sacrificed for biochemical studies. Immunoblot analyses indicated that membrane $G_s\alpha$ expression was significantly greater in the cerebellum, nucleus accumbens, VTA, and lateral septum of HAD compared to LAD rats (Table 1). LAD rats had greater $G_s\alpha$ expression in the frontal cortex (170 ± 6 vs. 100 ± 4) and hippocampus (130 ± 10 vs. 100 ± 4) compared to HAD rats (Table 1). The expression of the inhibitory G-protein, $G_i\alpha_2$ was reduced in the cerebellum of LAD rats compared to HAD rats (Table 1). GTP-γS-stimulated and forskolin-stimulated AC activity was also measured in membranes derived from these brain regions (Table 2). In general, HAD rats had slightly higher levels of membrane AC activity compared to LAD rats, although these differences reached statistical significance only in the nucleus accumbens and cerebellum (Table 2).

Correlation Between Initial Sensitivity to Alcohol and Adenylyl Cyclase Activity

Of the 270 correlations that were performed, 9% were found to be statistically significant. There was a significant

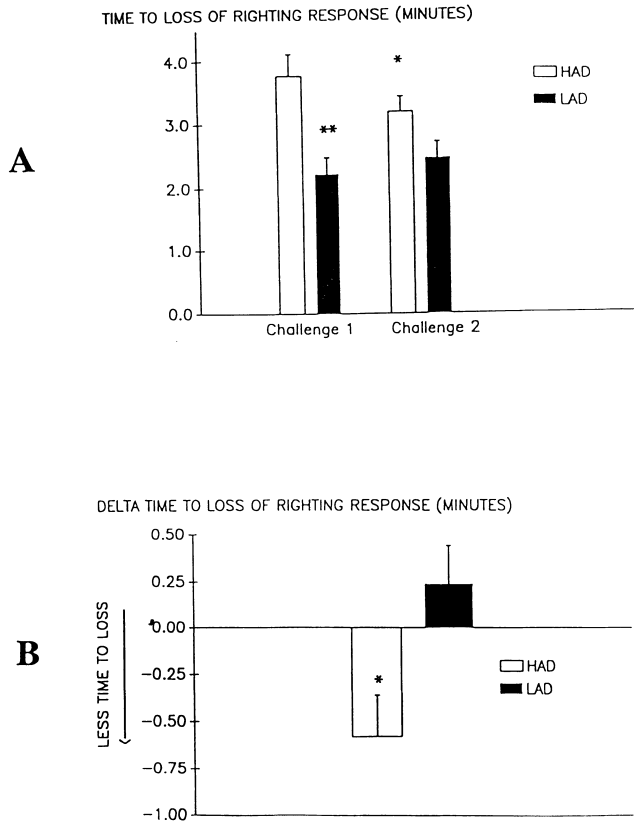

FIG. 1. (A) Mean (±SEM) latency to lose the righting response (RR) following each of two alcohol challenges (3.0 g/kg b.wt., IP) administered 1 day apart in rats of the HAD ($n = 8$) and LAD ($n = 8$) lines. $^{**}p = 0.003$ significant difference between the HAD and LAD lines after a single alcohol challenge; $^{*}p = 0.034$ significant difference between the first and the second alcohol challenge in rats of the HAD line. (B) Mean (±SEM) change in latency to lose the RR following the second alcohol challenge compared with the first in rats of the HAD and LAD lines. $^{*}p < 0.05$ significant difference between the first and the second alcohol challenge in rats of the HAD line.

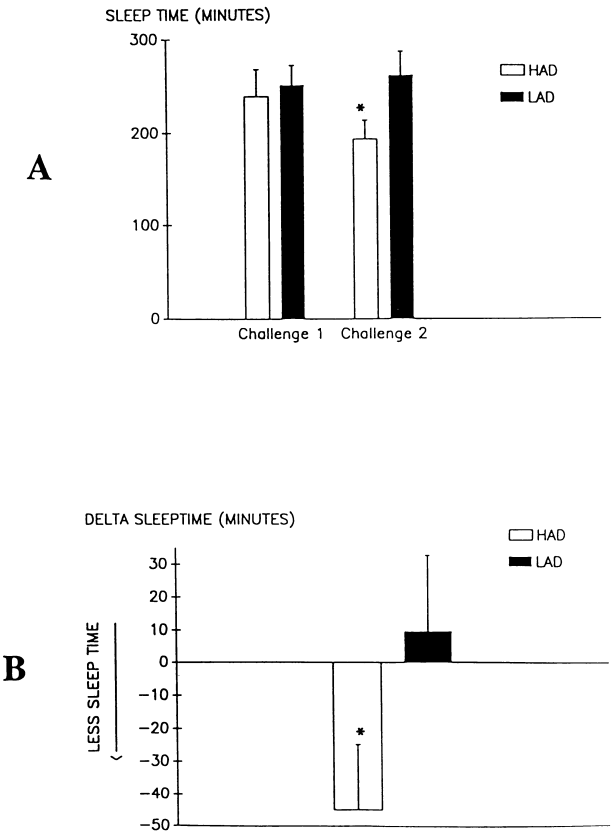

FIG. 2. (A) Mean (±SEM) duration of alcohol-induced sedation (sleep time) following each of two alcohol challenges (3.0 g/kg b.wt., IP) administered one day apart in rats of the HAD (n = 8) and LAD (n = 8) lines. $^{*}p = 0.059$ significant difference between the first and the second alcohol challenge in rats of the HAD line. (B) Mean (±SEM) change in sleep time following the second alcohol challenge compared with the first in rats of the HAD and LAD lines. $^{*}p < 0.05$ significant difference between the first and the second alcohol challenge in HAD rats.

TABLE 1
G PROTEIN EXPRESSION

Tissue	HAD	LAD	*p*-Value
$G_s\alpha$			
Cerebellum	100 ± 05	75 ± 07	<0.01
Nucleus accumbens	100 ± 04	80 ± 03	<0.02
VTA	100 ± 05	71 ± 09	<0.02
Lateral septum	100 ± 04	80 ± 06	<0.02
Hippocampus	110 ± 06	130 ± 10	<0.05
Frontal cortex	100 ± 04	170 ± 06	<0.001
$G_i\alpha_2$			
Cerebellum	100 ± 05	72 ± 08	<0.02
Nucleus accumbens	100 ± 06	100 ± 11	NS
VTA	100 ± 08	75 ± 12	NS
Lateral septum	100 ± 05	100 ± 06	NS
Frontal cortex	100 ± 18	72 ± 09	NS

Comparison of G protein expression in specific brain regions of HAD and LAD rats. Data is expressed relative to HAD rats. For this analysis, eight HAD and six to eight LAD animals were used. Data is expressed as mean ± SEM.

correlation between $G_s\alpha$ content in membranes derived from the hippocampus and frontal cortex and sensitivity to alcohol as measured by latency to lose the RR after a single alcohol challenge (Fig. 3A and 3C). Rats with the highest hippocampal AC activity and frontal cortical $G_s\alpha$ levels were more sensitive to, or rapidly affected by, the sedative properties of alcohol than were rats with lower $G_s\alpha$ levels. This correlation was reversed after alcohol exposure. Rats with the highest hippocampal and frontal cortical $G_s\alpha$ levels were less sensitive to the sedative effects of alcohol after the second alcohol challenge compared to the first (Fig. 3B and 3D).

$G_i\alpha_2$ is an inhibitory G-protein that can diminish membrane AC activity. HAD rats had higher levels of $G_i\alpha_2$ expression in the cerebellum compared to LAD rats (Table 1). There was a positive correlation between the amount of membrane $G_i\alpha_2$ expression and latency to lose RR after a single alcohol challenge (Fig. 4A). Rats with the highest $G_i\alpha_2$ expression in cerebellar membranes were the least sensitive to the sedative effects of alcohol. Moreover, rats with higher $G_i\alpha_2$ levels became more sensitive to the second alcohol challenge compared to the first (Fig. 4B).

Correlation Between Duration of Alcohol-Induced Sedation (Sleep Time) and Adenylyl Cyclase Activity

In LAD rats there was a negative correlation between the amount of forskolin-stimulated AC in the cerebellum and duration of alcohol-induced sedation (sleep-time) after a single alcohol challenge (Fig. 5A). A similar negative correlation was observed in the nucleus accumbens (Fig. 5B). When rats from the HAD and LAD lines were combined, a significant negative correlation was also found between the amount of forskolin-stimulated AC in the hippocampus and duration of alcohol-induced sedation (Fig. 5C). Furthermore, there was a significant positive correlation between membrane $G_s\alpha$ content in the lateral septum and the development of tolerance to the sedative effects of alcohol (Fig. 5D). Rats with the highest membrane $G_s\alpha$ levels were those that rapidly developed tolerance to the sedative effects of alcohol.

DISCUSSION

One approach to identifying traits that are associated with, and may be causally related to, high alcohol drinking is to determine how organisms with a genetic predisposition toward drinking differ from organisms without genetic risk. When making such a comparison it is useful to examine more than one population with demonstrable risk. A number of rodent lines that differ in genetic predisposition toward alcohol drinking have been derived using a selective breeding approach (7,8,25,26,28,29). Two of these lines, the alcohol-preferring (P) and the alcohol-nonpreferring (NP) lines have been well characterized, and several behavioral and neurochemical traits have been identified that are associated with high alcohol drinking in P rats (11,15,21,22,34,37,43–46). Two of these traits, low initial sensitivity to alcohol (15,18,25) and the rapid development of alcohol tolerance (15,37,38,45) are of theoretical interest because they may contribute mechanistically to high alcohol intake (9,13,15,16,36,42). In the present study the relationship between alcohol drinking behavior, sensitivity to the sedative properties of alcohol, and the development of tolerance to this alcohol effect was examined in rats selectively bred for high (HAD) or low (LAD) alcohol

TABLE 2
ADENYLYL CYCLASE ACTIVITY

	GTPγs			Forskolin		
Tissue	HAD	LAD	*p*-Value	HAD	LAD	*p*-Value
Cerebellum	81 ± 2	74 ± 2	<0.02	179 ± 6	181 ± 6	NS
Nuc acc	100 ± 6	46 ± 17	<0.01	180 ± 23	81 ± 19	<0.01
VTA	55 ± 14	27 ± 7	NS	84 ± 27	42 ± 11	NS
Lat septum	65 ± 6	53 ± 2	NS	39 ± 4	38 ± 3	NS
Front cortex	82 ± 10	56 ± 11	NS	238 ± 30	162 ± 41	NS
Hippocampus	74 ± 100	78 ± 4	NS	151 ± 10	144 ± 7	NS
Hypothalamus	104 ± 6	112 ± 3	NS	112 ± 7	113 ± 6	NS
Ant pituitary	35 + 4	40 + 7	NS	51 + 4	62 + 8	NS
Ant striatum	245 + 22	218 + 24	NS	—	—	NS

Comparison of adenylyl cyclase activity between specific brain regions of HAD and LAD rats. Activity is expressed as pmol/mg membrane protein/min following stimulation with either 10 μM GTPγs or 1 μM forskolin. For this analysis, eight HAD and six to eight LAD animals were used. Data is expressed as mean ± SEM.

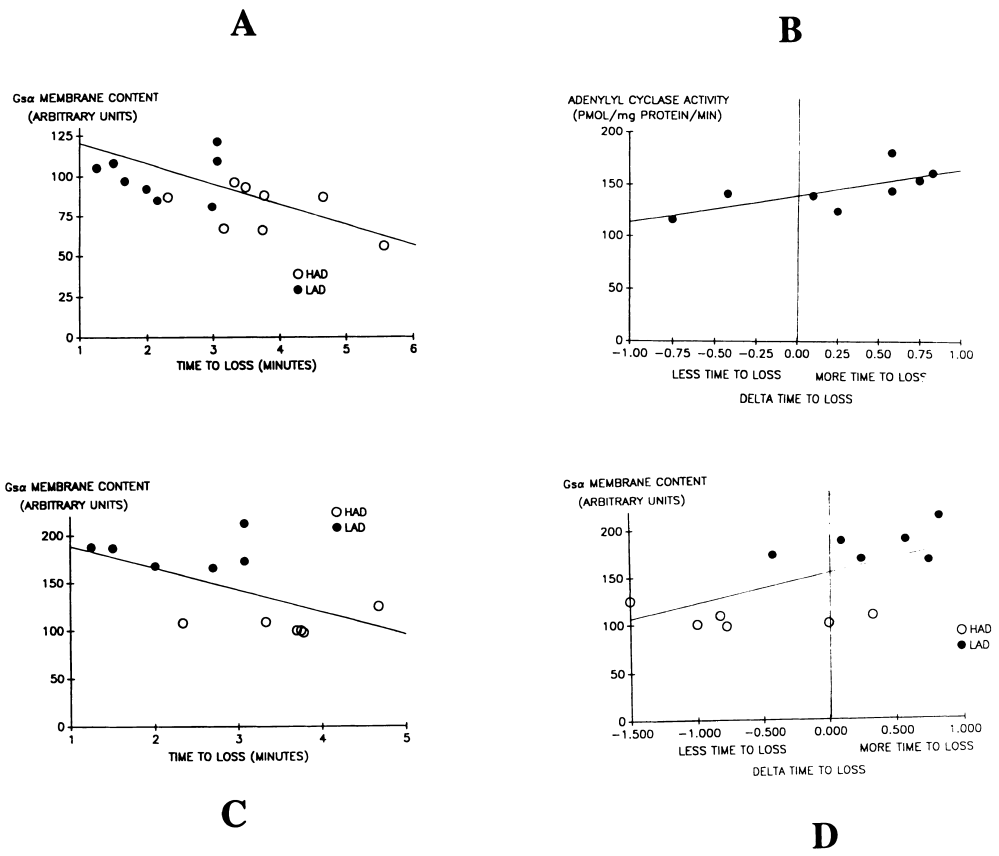

FIG. 3. (A) Correlation between hippocampal membrane $G_s\alpha$ levels and initial sensitivity to alcohol as measured by latency to lose the righting response after a single alcohol challenge (challenge 1) $r = 0.64$, $p < 0.04$. Eight animals were used per group. (B) Correlation between forskolin-stimulated adenylyl cyclase activity in LAD ($n = 8$) hippocampal membranes and change in latency to lose the righting response between the first and second alcohol challenges. $r = 0.7$, $p < 0.04$. (C) Correlation between frontal cortex membrane $G_s\alpha$ levels and initial sensitivity to alcohol as measured by latency to lose the righting response after a single alcohol challenge (challenge 1). $r = 0.58$, $p < 0.02$. Six animals were used per group. (D) Correlation between frontal cortex membrane $G_s\alpha$ levels and change in latency to lose the righting response between the first and second alcohol challenges. $r = 0.7$, $p < 0.02$. Six animals were used per group.

drinking. As has previously been reported in the P/NP lines, a positive association was found between low initial sensitivity to alcohol, the rapid development of alcohol tolerance, and high alcohol intake in rats of the HAD and LAD lines. Similar associations have also been reported in other lines and strains of rodents (9,16,36,42). In a broad sense, the results of the present study provide evidence for strong test-retest reliability of the association between alcohol drinking and these alcohol related traits. The generality of the association between low sensitivity to alcohol, the rapid development of alcohol tolerance, and high alcohol drinking strongly suggests that these traits may be mechanistically related to the alcohol drinking phenotype.

Currently, little is known regarding the neural circuitry and biochemical processes that underlie sensitivity to alcohol and the rapid development of alcohol tolerance. We hypothesize that the level of membrane AC activity may determine the degree of alcohol-induced activation of the AC signal transduction system, which in turn, may contribute to the magnitude of certain alcohol-induced responses. This view is suggested by the following findings: 1) the AC signal cascade is a ubiquitous system in the CNS, 2) it is particularly sensitive to alcohol exposure and, 3) it modulates membrane, cytosolic, and genomic events. We posit that the level of membrane adenylyl cyclase (AC) activity in certain alcohol-sensitive brain regions may contribute to individual differences in sensitivity to alcohol and differences in the rate of tolerance development, both of which are found to exhibit significant variation in the general population. To begin testing this hypothesis we compared membrane AC activity and G-protein expression in discrete brain regions of rats that differ in sensitivity to the sedative effects of alcohol and in the development of tolerance to this alcohol effect.

Hormone-sensitive AC signal transduction requires the interaction of at least three types of membrane proteins: specific receptors, guanine nucleotide-binding regulatory proteins (G proteins), and catalytic proteins. G proteins are a superfamily of proteins that regulate a diverse array of cellular functions, including transducing transmembrane signals, directing fidelity of protein synthesis, guiding vesicular transport through the cytoplasm, and controlling cellular growth and differentiation. AC responds to signals that arise from interactions of hormones,

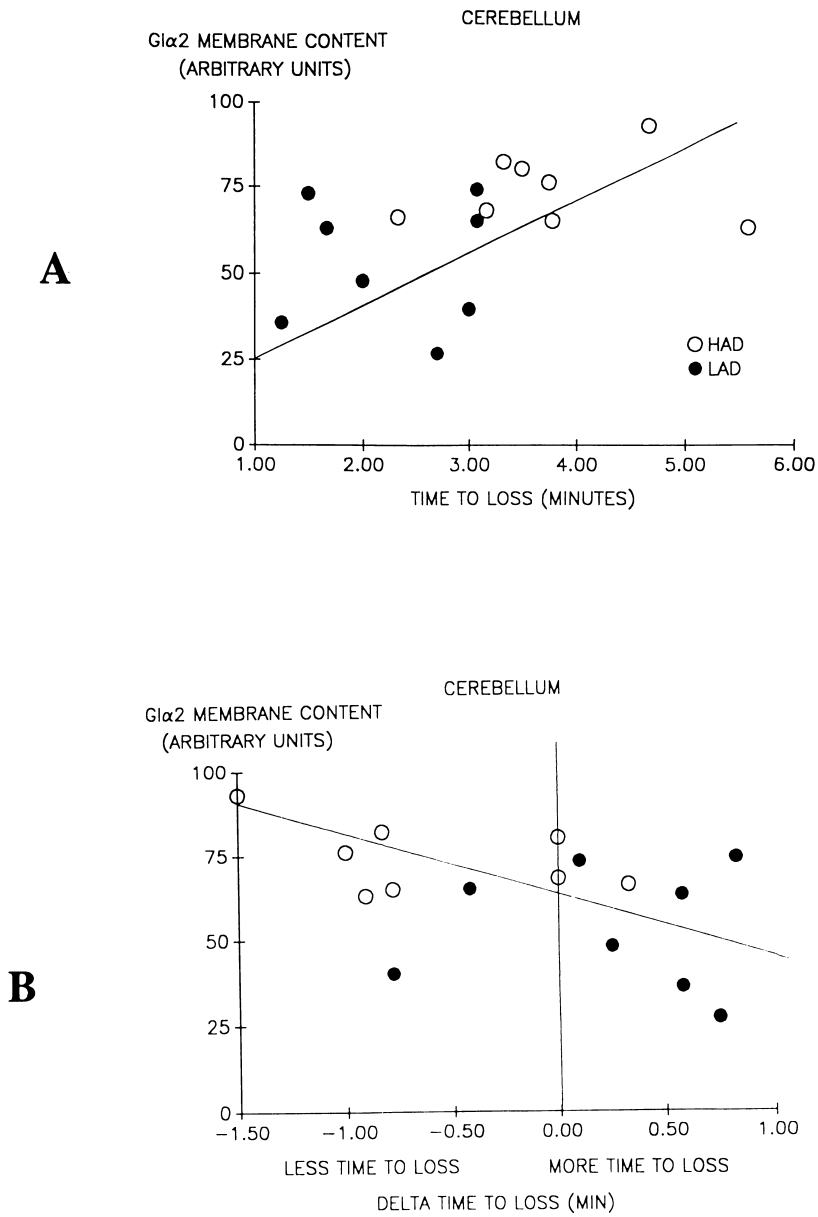

FIG. 4. (A) Correlation between cerebellar membrane $G_i\alpha_2$ levels and latency to lose the righting response after a single alcohol challenge. $r = 0.49$, $p < 0.05$. Eight animals were used per group. (B) Correlation between cerebellar membrane $G_i\alpha_2$ levels and change in latency to lose the righting response between the first and second alcohol challenges. $r = 0.57$, $p < 0.05$. Eight animals were used per group.

neurotransmitters, and other agonists with specific cell surface receptors through the action of G proteins. Receptors communicate with the AC system through at least two classes of G proteins; Gs stimulates AC activity, whereas Gi inhibits AC activity. Stimulation of AC enhances cAMP formation, which subsequently acts upon cAMP-dependent protein kinases and ultimately leads to physiologic expression of the agonist.

The AC signal transduction pathway may represent a potential marker for vulnerability to alcoholism (6,40,47). In the presence of guanine nucleotides, acute alcohol exposure enhances AC activity (12). Alcohol alters the rate of activation of Gs and enhances the action of $G_s\alpha$ with guanine nucleotides (12). In response to chronic alcohol exposure adaptive changes occur in the AC system that are associated with reduced AC activity as has been demonstrated both in vivo (rats) and in vitro (cultured cells) (12). These findings suggest that $G_s\alpha$ is an important site of action for alcohol within the receptor-coupled AC complex.

Evidence is accumulating that the response of the AC system to alcohol is, in part, heritable. This view is supported by the finding that individual variability in platelet membrane fluoride-stimulated AC activity is largely genetic (5). In the pituitary, the ability of alcohol to regulate expression of $G_s\alpha$ has also been shown to be influenced by genetic factors (51).

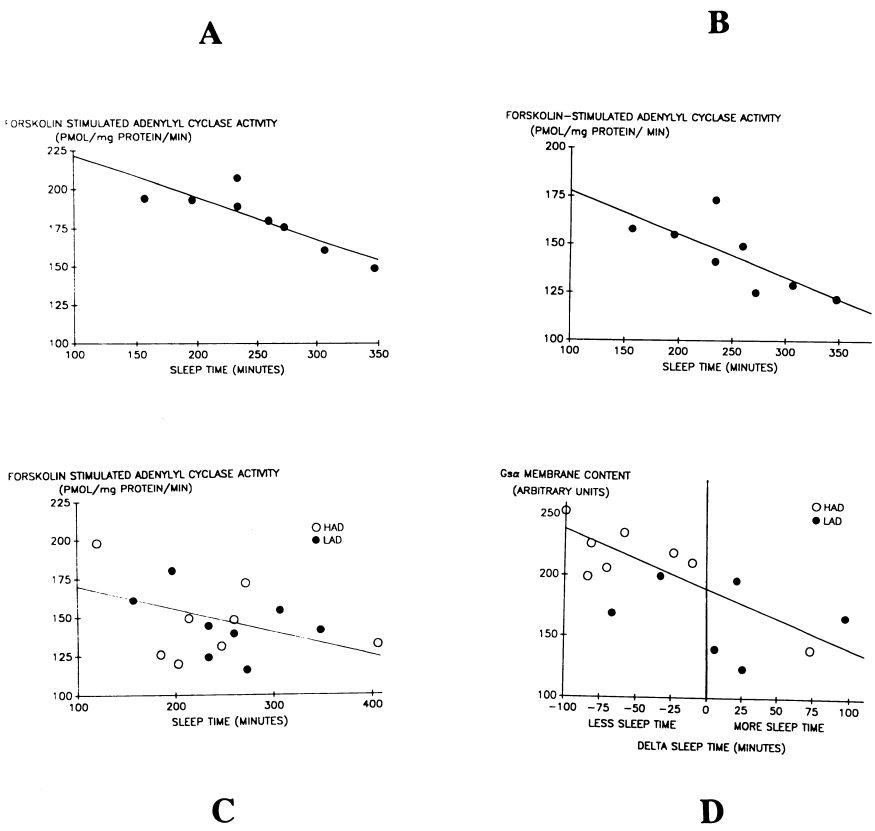

FIG. 5. (A) Correlation between forskolin-stimulated adenylyl cyclase activity in the cerebellum and sleep time following a single alcohol challenge in rats of the LAD ($n = 8$) line. $r = 0.58$, $p < 0.01$. (B) Correlation between forskolin-stimulated adenylyl cyclase activity in nucleus accumbens membranes and sleep time following a single alcohol challenge in rats of the LAD ($n = 8$) line. $r = 0.77$, $p < 0.02$. (C) Correlation between forskolin-stimulated adenylyl cyclase activity in hippocampal membranes and sleep time following a single alcohol challenge in rats of the HAD and LAD lines. $r = 0.46$, $p < 0.03$. Eight animals were used per group. (D) Correlation between lateral septum $G_s\alpha$ levels and change in sleep time between the first and second alcohol challenges in rats of the HAD ($n = 8$) and LAD ($n = 6$) lines. $r = 0.76$, $p < 0.01$.

Abnormalities in AC activity have been found in peripheral blood lymphocytes and platelets isolated from alcoholic men (6,40,48). The fact that these abnormalities are present in platelets even after years of abstinence and in lymphocytes maintained in culture suggests that an underlying abnormality in the AC system in alcoholic men may predate the onset of alcoholism. Moreover, we have recently shown that nonalcoholic offspring of alcoholics have higher levels of $G_s\alpha$ expression in erythrocyte and lymphocyte membranes than do nonalcoholic offspring of nonalcoholics (20). Taken together, these results suggest that AC activity is, in part, genetically determined and may be important in defining individual differences in alcohol vulnerability. There are also several reasons for hypothesizing that $G_s\alpha$ may play an important role in modulating tissue sensitivity to alcohol and biological vulnerability to alcohol and other drugs of abuse. $G_s\alpha$ is an essential component of the AC signal transduction system. In the central nervous system alcohol, opiates, and cocaine perturb AC activity by altering the expression of key G proteins (31, 32,49). In the mesolimbic dopamine reward pathway, modulation of the AC system, via D_1- and D_2-dopamine receptors, is influenced by alcohol and other drugs of abuse (3).

In the present study a positive correlation was found between $G_s\alpha$ content in membranes derived from the frontal cortex and hippocampus of rats and sensitivity to alcohol-induced sedation. Rats with the highest $G_s\alpha$ levels were more rapidly affected by the sedative properties of alcohol than were rats with lower $G_s\alpha$ levels. Interestingly in these same brain regions, rats with higher $G_s\alpha$ expression also developed a more marked degree of behavioral tolerance to this ethanol-induced event (e.g., loss of righting response) compared to animals with lower levels of $G_s\alpha$ expression. This suggests that in certain brain areas high $G_s\alpha$ expression may predict increased responsiveness to initial ethanol exposure and may also predict the more rapid development of tolerance. In our model, initial sensitivity and tolerance may be part of a signal transduction continuum that is ultimately expressed at the behavioral level. For example, it is conceivable that ethanol has a more pronounced effect on the adenylyl cyclase signal transduction pathway in tissues with high levels of expression of the stimulatory G protein, $G_s\alpha$. In this model, enhanced sensitivity to ethanol induces a marked activation of cAMP-dependent kinases (e.g., receptor kinases and/or protein kinase A) compared to tissue with low sensitivity to ethanol. Greater ac-

tivation of the kinases results in phosphorylation events that downregulate or desensitize the adenylyl cyclase cascade, reducing the effects of a subsequent ethanol exposure. Therefore, enhanced sensitivity leads to greater activation of the adenylyl cyclase pathway, which then induces a more rapid desensitization of the pathway. In this model, desensitization of the signal transduction pathway is the biochemical event underlying the development of behavioral tolerance to ethanol.

Correlating G-protein expression (specifically G proteins that modulate adenylyl cyclase) with behavior provides the first level of evidence to implicate adenylyl cyclase signal transduction in mediating certain ethanol-induced behaviors. The correlations are interesting but open to multiple interpretations. We speculate that a significant correlation between an ethanol-induced behavior and $G_s\alpha$ expression has a different meaning than does a significant correlation between an ethanol-induced behavior and Gi expression. The correlations have different meanings because Gs and Gi play different roles within the adenylyl cyclase cascade. For example, the magnitude of basal and stimulated membrane adenylyl cyclase activity is, in part, the net sum of the stimulatory and inhibitory arms of the signal transduction cascade. $G_s\alpha$ (and the receptors that couple to Gs) represent the stimulatory arm of the pathway whereas G_i2_α (and the receptors that couple to G_i2_α) is a major component of the inhibitory arm of the pathway. In certain tissue (e.g., frontal cortex and hippocampus) loss of the righting response (RR) correlated with $G_s\alpha$ expression but not with Gi expression; and in other tissue (e.g., cerebellum), RR correlated with Gi but not Gs. It is plausible that a correlation between G protein expression in a specific brain region with an ethanol-induced behavior tells us something about the neurotransmitters and/or receptors involved in mediating that ethanol-induced behavior. For example, a positive correlation between time to loss of RR with Gi expression in the cerebellum may indicate that the ethanol-induced behavior is mediated by neurotransmitter and/or receptors that utilize the inhibitory arm of the adenylyl cyclase pathway. In contrast, a positive correlation between change in latency to lose the RR and $G_s\alpha$ expression in the frontal cortex may indicate that this ethanol-induced behavior is modulated by neurotransmitters/receptors utilizing the stimulatory arm of the pathway. Such correlations allow for the design of future experiments that can effectively test the hypothesis that the level of G-protein expression may predict the magnitude of certain behavioral responses to ethanol.

In summary, G-protein expression and AC activity in specific brain regions were found to differ in rats of the HAD and LAD lines and were correlated with sensitivity to the sedative effects of alcohol and with the rapid development of alcohol tolerance in these selectively bred rodent lines. The results support the view that sensitivity to the sedative properties of alcohol and the development of tolerance to this alcohol effect may be mediated in part through the AC signal transduction system.

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