0026-895X/93/020158-09$03.00/0

MOLECULAR PHARMACOLOGY, 43:158-166

Ethanol Differentially Regulates Guanine Nucleotide-Binding Protein α Subunit Expression in NG108-15 Cells Independently of Extracellular Adenosine

ROBERT J. WILLIAMS, MARK A. VEALE, PETER HORNE, and EAMONN KELLY
Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK
Received July 2, 1992; Accepted November 10, 1992

SUMMARY

Recent work has suggested that chronic ethanol treatment induces heterologous desensitization of adenylate cyclase in a number of cell lines maintained in culture and that this phenomenon is mediated by adenosine. It has been proposed that ethanol induces the accumulation of extracellular adenosine, which then down-regulates the $G_{s\alpha}$ protein and leads to heterologous desensitization. Here we investigated the effects of chronic ethanol treatment on the expression of $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$, as well as cAMP signal transduction, in NG108-15 cells and further examined the role of adenosine in mediating these effects. Pretreatment of NG108-15 cells with 200 mM ethanol for 2 days reduced membrane levels of $G_{s\alpha}$ and $G_{i\alpha}$ and increased those of $G_{o\alpha}$. However, ethanol did not reduce the levels of $G_{s\alpha}$ and $G_{i\alpha 2}$ mRNA in these cells. The ability of ethanol to alter α subunit expression was not reversed by removal of extracellular adenosine and could not be mimicked by an adenosine agonist. Chronic ethanol treatment increased both basal and agonist-stimulated cAMP accumulation in NG108-15 cells. Whereas the increase in basal cAMP was abolished by acute addition of adenosine deaminase, the increase in agonist-stimulated cAMP accumulation was not. Morphological examination of the cells indicated that ethanol inhibited cell division and promoted the apparent differentiation of the cells. These results indicate that ethanol induces complex alterations in guanine nucleotide-binding protein α subunit expression and cAMP signal transduction in NG108-15 cells and that it is unlikely that these effects are mediated simply by adenosine.

Recently, a number of studies have attempted to define the cellular and molecular consequences of chronic ethanol treatment (reviewed in Ref. 1). The acute cellular actions of ethanol have also been investigated (2), but chronic studies are perhaps of more interest because of their possible relationship to alcoholism. Recent work has focused on the interaction between ethanol and the adenylate cyclase signaling system in cells. It was found that chronic ethanol treatment induced heterologous desensitization in NG108-15 and other cell lines (3, 4). Subsequent work indicated that ethanol produced a selective decrease in $G_{s\alpha}$ mRNA and hence $G_{s\alpha}$ protein in NG108-15 cells (5). G_s is a heterotrimer composed of α, β, and γ subunits and is the regulatory G protein that couples stimulatory receptors to adenylate cyclase (6). A selective loss of $G_{s\alpha}$ would provide the basis for the heterologous desensitization observed in NG108-15 cells. In support of such a selective action, in this cell line chronic ethanol treatment was reported not to affect levels of $G_{i\alpha}$ (7), a subunit of the G_i protein that couples inhibitory receptors to adenylate cyclase (6). Further work indicated that the basis of the chronic action of ethanol is the inhibition of adenosine uptake by ethanol in NG108-15 cells (4, 8). Adenosine is released by these cells, so that inhibition of uptake would lead to the accumulation of extracellular adenosine and, hence, prolonged stimulation of adenosine A_2 receptors that exist on these cells (9). This in turn would lead to prolonged increases in intracellular cAMP and, hence, to heterologous desensitization of adenylate cyclase-stimulating receptors (4).

On the other hand we have previously shown that prolonged treatment of NG108-15 cells with the adenosine agonist NECA leads only to homologous desensitization of A_2 receptor-activated adenylate cyclase activity; prostacyclin receptor-stimulated and forskolin-stimulated adenylate cyclase activities are unchanged (9). Furthermore, we have recently shown that in NG108-15 cells chronic treatment with forskolin or the cAMP-dependent protein kinase A activator 8-bromo-cAMP does not affect G_s function (10). Also, a number of recent studies indicate that chronic ethanol treatment has multiple actions on cellular processes; in NG108-15 cells ethanol increases the expression

This study was supported by the Wellcome Trust. M.A.V. is a MRC scholar.

ABBREVIATIONS: $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$, guanine nucleotide-binding regulatory protein α subunits; SDS, sodium dodecyl sulfate; NECA, 5′-(*N*-ethylcarboxamido)adenosine; G protein, guanine nucleotide-binding protein; TBS, Tris-buffered saline; TTBS, Tween-containing Tris-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; kb, kilobase(s).

of the Hsc70 stress protein (11) and increases protein kinase C activity (12), whereas in NIE-115 cells ethanol increases tyrosine hydroxylase gene expression (13). In light of these findings we decided to investigate in more detail the effects of chronic ethanol treatment on G protein α subunit expression and cAMP signal transduction in NG108-15 cells. We report that ethanol alters the expression of at least three types of α subunit and enhances agonist-stimulated cAMP accumulation but that these effects are unlikely to be mediated by adenosine.

Materials and Methods

Reagents. [^{32}P]dCTP (3000 Ci/mmol) and ^{125}I-labeled goat anti-rabbit IgG (2–10 μCi/μg) were purchased from New England Nuclear, whereas [8-^{3}H]cAMP (26 Ci/mmol) was obtained from Amersham International. Antibodies to the carboxyl-terminal decapeptides of $G_{s\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$ were obtained from BioMac Ltd. Oligolabeling reagents were purchased from Pharmacia, and partial cDNA sequences of $G_{s\alpha}$ and $G_{i\alpha 2}$ were generously provided by Dr R. Randall Reed, John Hopkins University School of Medicine (Baltimore, MD). Iloprost was a generous gift from Schering AG (Berlin, Germany). All other reagents and drugs were obtained from Sigma Chemical Co. and BDH Chemicals Ltd.

Cell culture. NG108-15 neuroblastoma × glioma hybrid (and NCB-20) cells were cultured in Dulbecco's modified Eagle's medium containing 6% (v/v) fetal calf serum and supplemented with 1 μM aminopterin, 100 μM hypoxanthine, and 16 μM thymidine. Culture flasks (25 cm^2) were maintained at 37° in a humidified atmosphere of 6% CO_2/94% air. Cells (passages 22–32) were grown in the presence or absence of ethanol (10–200 mM), with or without adenosine deaminase (1 unit/ml) as required. Ethanol treatments were begun 3–4 days after splitting, when the cells were preconfluent. Medium was changed at 24 hr and the relevant ethanol/adenosine deaminase concentrations were replaced. This protocol has previously been reported not to alter significantly the ethanol concentration of the medium between changes (3). Cells were harvested in 5 ml of phosphate-buffered saline and pelleted by centrifugation at 200 × g for 2 min. The resultant pellets were washed once in 10 ml of phosphate-buffered saline at 37° and either used immediately or frozen at −80° until required. Morphological examination of cells was undertaken with a Nikan DIAPHOT-TMD inverted microscope. Photography was performed using 35-mm Ilford FP4 black and white film. Cell proliferation curves for NG108-15 cells were obtained by thinly seeding cells in 25-cm^2 culture flasks and counting cell numbers in 1-mm^2 grid areas of the monolayer at various later times.

Membrane preparation and immunoblotting. Cell pellets were resuspended in 1 ml of ice-cold 25 mM Tris, 0.3 M sucrose, pH 7.5, and were lysed with 25 full strokes in a Teflon-glass hand-held homogenizer. Lysates were then centrifuged at 500 × g for 15 min at 4° to remove nuclei and unbroken cells. The supernatant was then centrifuged at 16,000 × g for 30 min at 4° and the resultant pellets were washed in 1 ml of ice-cold TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The final washed membrane pellet was resuspended in 100 μl of TE and stored at −80°. The membrane protein content was determined by the method of Bradford (14), using a bovine serum albumin standard. SDS-polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (15), using a 4% stacking gel and 12% resolving gel. Membrane samples (60 μg/lane) were solubilized and denatured by boiling for 2 min in an equal volume of 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50 mM Tris, pH 6.5, 10% glycerol, 0.005% (w/v) bromophenol blue. Separated proteins were transferred to Hybond C nitrocellulose membranes (Amersham) by overnight electroblotting. The membranes were then incubated in blocking buffer (20 mM Tris, 500 mM NaCl, pH 7.5) (TBS), containing 4% skim milk powder, for 30 min at room temperature with constant agitation, followed by two 5-min rinses in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Blots were then incubated with the appropriate anti-G protein α subunit antibody (1/500 dilution of stock) in TTBS, containing 1% skim milk powder (antibody buffer), overnight at room temperature with constant agitation. The blots were rinsed in TTBS and then incubated with ^{125}I-labeled anti-rabbit IgG (0.1 μCi/ml) in antibody buffer for 90 min. Finally, the blots were washed for 20 min in TTBS and 10 min in TBS, air dried, and subjected to autoradiography using Amersham Hyperfilm. Autoradiographs were stored at −80° for 2–7 days before development. Bands were quantified by being cut from the blot and γ counted.

Analysis of mRNA by Northern blotting. Total RNA was isolated from NG108-15 cells by the rapid extraction procedure of Stallcup and Washington (16). After denaturation of the RNA with 1 M glyoxal at 50° for 1 hr, it was resolved in 1.2% agarose gels and transferred to Hybond C filters by capillary blotting. rRNA (18 and 28 S) from calf liver was also subjected to agarose gel electrophoresis to determine the size of hybridized bands. The filters were prehybridized at 42° for 2 hr in 50% deionized formamide, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 1 M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, 0.05 M Tris·HCl, pH 7.5, 100 μg/ml denatured salmon sperm. The filters were then prehybridized at 42° for 16 hr in the aforementioned solution containing ^{32}P-labeled *Eco*RI fragments of common $G_{s\alpha}$ or $G_{i\alpha 2}$. The probes were prepared by oligolabeling of cDNA using hexadeoxynucleotide primers and [α-^{32}P]dCTP. After hybridization, the filters were washed with 2× standard saline citrate (0.3 M NaCl, 30 mM trisodium citrate), 1% SDS, at 65° for 1 hr. Blots were exposed to Amersham Hyperfilm at −80° for 72 hr.

cAMP determinations. cAMP levels in NG108-15 cells were measured using a protein-binding assay (17). Control and ethanol-pretreated cells were washed three times in phosphate-buffered saline at 37°, and the pellets were resuspended in ice-cold Krebs-Ringer-HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 10 mM HEPES, 1 mg/ml bovine serum albumin, and 100 μM Ro20 1724 as phosphodiesterase inhibitor, pH 7.4). Aliquots (100 μl, containing about 250–500 μg of protein) of the cell suspension were then pipetted into tubes on ice, to which were then added 100 μl of buffer containing relevant drug concentrations, and the tubes were then placed in a shaking water bath at 37° for 10 min (accumulation of cAMP over this time period is linear). At the end of this time 20 μl of ice-cold 100% trichloroacetic acid were added to each tube and the tubes were placed on ice for 20 min. Precipitated protein was pelleted by centrifugation at 2900 × g for 20 min at 4°, and 100 μl of the supernatant were added to 100 μl of 1 M NaOH and 1 ml of 50 mM Tris, 4 mM EDTA, pH 7.4 (TE buffer). One hundred microliters of this solution were then added to fresh tubes containing 50 μl of TE buffer, 100 μl of [^{3}H]cAMP in TE buffer (about 40,000 cpm), and 100 μl of binding protein (to give a final concentration of ~750 μg/ml; prepared from bovine adrenal cortex). Tubes containing 100 μl of standard concentrations of cAMP (0.125–10 pmol) were used to construct a standard curve. After 2–3 hr of incubation 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg/ml final concentration) and bovine serum albumin (2 mg/ml final concentration) were added, and 15 min later the tubes were centrifuged at 2900 × g for 20 min at 4°. The resulting supernatant was poured into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (GraphPAD) and sample values were determined. The protein content of cell suspensions was determined (14) and the results were expressed as pmol of cAMP/min/mg of protein.

Experimental design and statistics. In all experiments drug-treated cells were grown in parallel with untreated controls. Transformed data (expressed as percentage of control) were tested for statistical significance (at the 5% level) by an unpaired Mann-Whitney test. cAMP data were tested for statistical significance by the Student's t test.

Results

In membrane preparations of NG108-15 cells subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting,

the anti-$G_{s\alpha}$ antibody identified a major band at 45 kDa, the anti-$G_{i\alpha}$ antibody a band at 40 kDa, and the anti-$G_{o\alpha}$ antibody a band at 39 kDa (Fig. 1). The effects of pretreatment of NG108-15 cells with 200 mM ethanol for 48 hr on G protein α subunit expression are also shown in Fig. 1. Levels of $G_{s\alpha}$, when expressed per unit of membrane protein, were reduced by 24.5 ± 2.0% and $G_{i\alpha}$ by 34.2 ± 1.6%, but $G_{o\alpha}$ expression was increased by 43.1 ± 3.3% (means ± standard errors). Figs. 2 and 3 show that these were concentration-dependent changes; the apparent EC_{50} values for the ethanol-induced decreases in $G_{s\alpha}$ and $G_{i\alpha}$ lay between 50 and 100 mM. In a single experiment ethanol also concentration-dependently increased $G_{o\alpha}$ expression (Fig. 4). The changes in α subunit expression were not restricted to the NG108-15 cell line; in NCB-20 cells ethanol (200 mM; 48 hr) also reduced the expression of $G_{s\alpha}$ and $G_{i\alpha}$ (Fig. 5). Northern analysis of mRNA extracted from NG108-15 cells indicated that the $G_{s\alpha}$ and $G_{i\alpha 2}$ cDNA probes hybridized with a single band of 1.8–1.9 kb in each case (Fig. 6). Treatment of cells with ethanol (200 mM; 48 hr) did not reduce levels of $G_{s\alpha}$ and $G_{i\alpha 2}$ mRNA; indeed, our results suggested moderate increases in these transcripts.

We next investigated the involvement of adenosine in the ethanol-induced changes in G protein α subunit expression. NG108-15 cells were pretreated with ethanol, adenosine deaminase (1 unit/ml; 48 hr), or a combination of these two agents. Adenosine deaminase alone did not affect expression of $G_{s\alpha}$, $G_{i\alpha}$, or $G_{o\alpha}$ (data not shown); neither did it reverse the effects of ethanol on the expression of these subunits (Fig. 7). The adenosine receptor agonist NECA (10 μM; 48 hr) also did not affect the expression of $G_{s\alpha}$ (Fig. 8), $G_{i\alpha}$, or $G_{o\alpha}$ (data not shown).

Basal cAMP accumulation was increased (by 74%) after chronic ethanol treatment (Fig. 9A). Chronic addition of adenosine deaminase (1 unit/ml) in combination with ethanol did not reverse this effect; rather, basal levels of cAMP accumulation were further increased. Acute addition of adenosine deaminase (in the 10-min incubation) reduced basal cAMP levels in control cells by 23% (control basal accumulation was 47.4 ± 6.8 pmol of cAMP/min/mg of protein and control basal accumulation in the presence of 1 unit/ml adenosine deaminase was 36.5 ± 7.2 pmol of cAMP/min/mg of protein; $p < 0.05$) but completely abolished the increase in basal of cAMP due to chronic ethanol treatment or the combination of chronic ethanol and chronic adenosine deaminase treatment (compare Fig. 9, A and B). The increases in cAMP accumulation in NG108-15 cells due to the adenosine receptor agonist NECA and the prostacyclin receptor agonist iloprost were enhanced by chronic ethanol treatment. This increase due to ethanol was not reversed by chronic treatment of cells with adenosine deaminase (Fig. 9A). Whereas the increase in basal cAMP levels due to chronic ethanol treatment was abolished by acute adenosine deaminase treatment, the increase in agonist-stimulated cAMP accumulation due to chronic ethanol treatment or the combination of chronic ethanol and chronic adenosine deaminase treatment was unaffected by acute adenosine deaminase treatment (Fig. 9B).

During membrane preparation, it became evident that cell pellets from ethanol-pretreated cells were always smaller than controls. We, therefore, undertook a morphological examination of the cells (Fig. 10). Ethanol-pretreated flasks contained fewer cells, which appeared to be in a more differentiated state

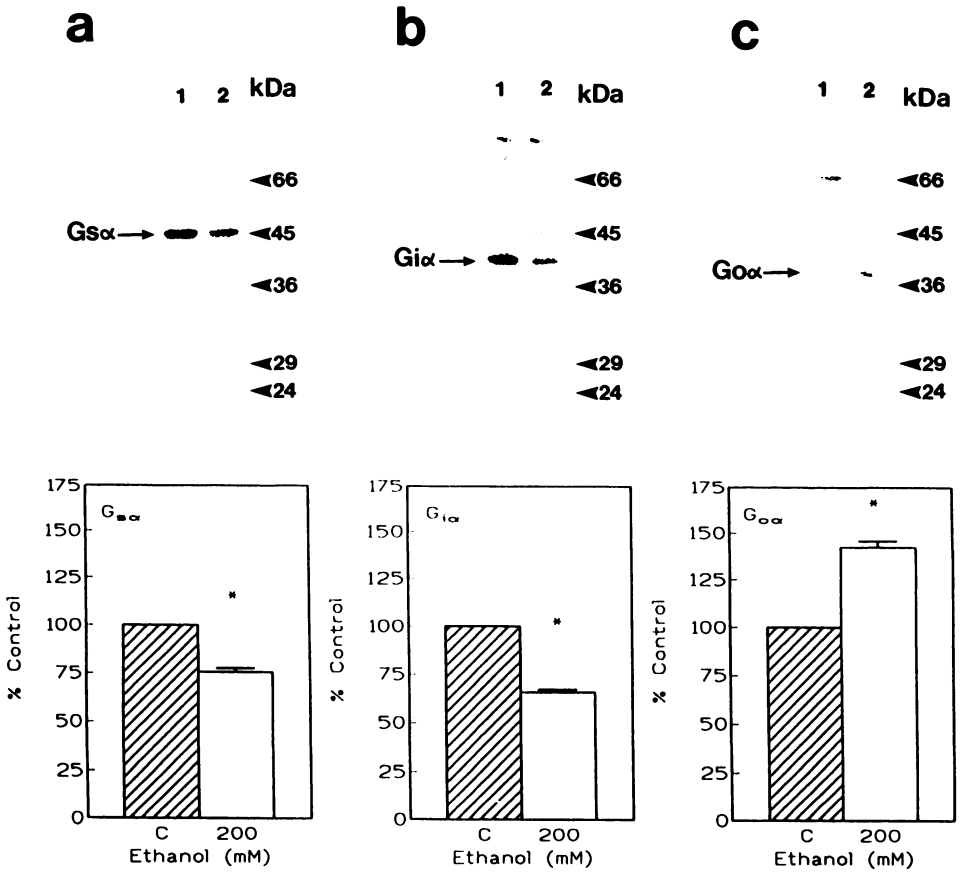

Fig. 1. Effect of ethanol treatment (200 mM; 48 hr) on the expression of G protein α subunits in membranes of NG108-15 cells. Representative autoradiographs are shown for $G_{s\alpha}$ (a), $G_{i\alpha}$ (b), and $G_{o\alpha}$ (c) in control (*lanes 1*) and ethanol-pretreated (*lanes 2*) cells. Bar graphs (means ± standard errors) show effects of ethanol on expression of $G_{s\alpha}$ (eight experiments), $G_{i\alpha}$ (five experiments), and $G_{o\alpha}$ (four experiments). In each case bands were quantified by cutting out and radioactivity counting. Results are expressed as a percentage of control (▨) (taken as 100% in each experiment). *, Ethanol pretreatment significantly altered the levels of each G protein α subunit, $p < 0.01$.

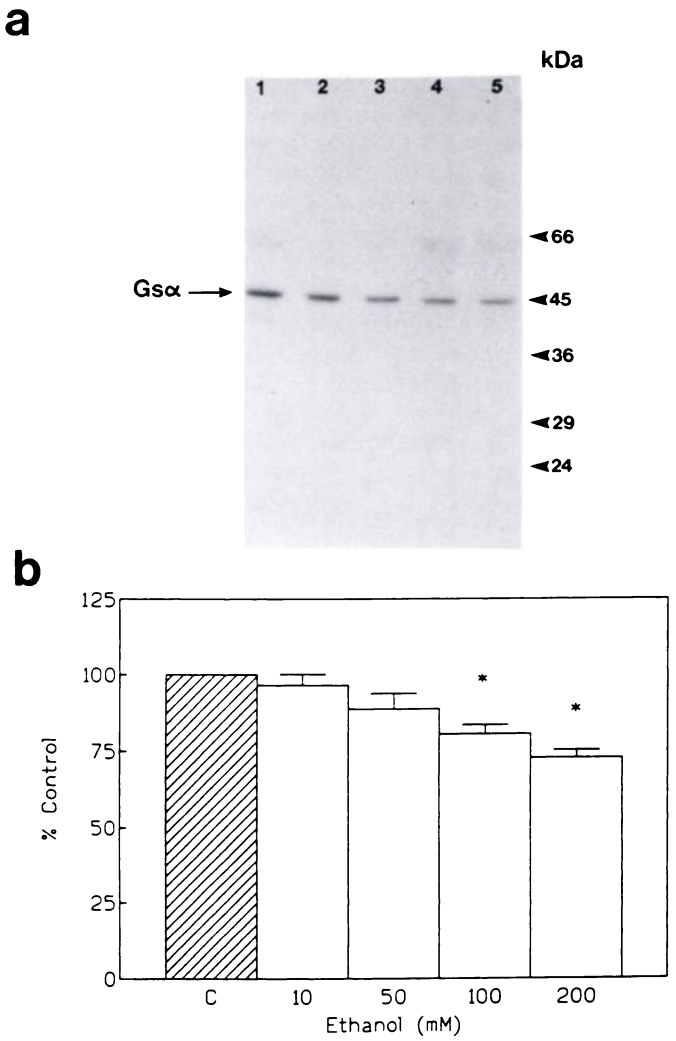

Fig. 2. Concentration-dependent loss of $G_{s\alpha}$ in NG108-15 cell membranes produced by 48-hr pretreatment with ethanol (10–200 mM). a, Representative autoradiograph for control (*lane 1*) and 10 mM (*lane 2*), 50 mM (*lane 3*), 100 mM (*lane 4*), and 200 mM (*lane 5*) ethanol-pretreated cells. b, Bar graph (means ± standard errors, four experiments), showing effects of ethanol (10–200 mM) on $G_{s\alpha}$ expression. Results are expressed as a percentage of control (▨) (taken as 100% in each experiment). *, Ethanol significantly reduced $G_{s\alpha}$ levels at 100 and 200 mM, $p < 0.05$.

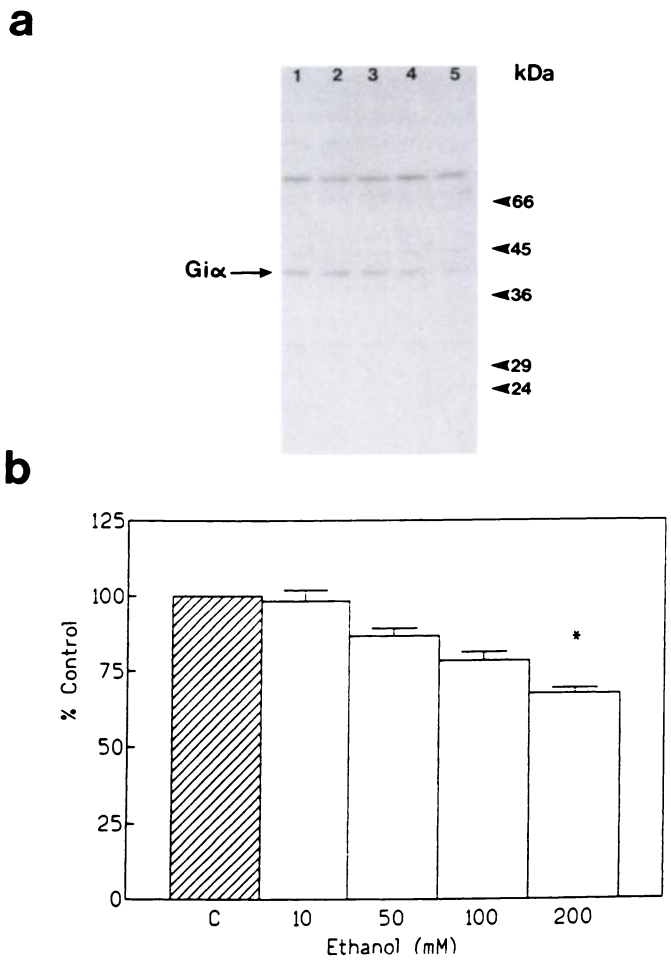

Fig. 3. Concentration-dependent loss of $G_{i\alpha}$ in NG108-15 cell membranes produced by 48-hr pretreatment with ethanol (10–200 mM). Conditions and *lanes* are as in Fig. 2. *, Ethanol (three experiments) significantly reduced $G_{i\alpha}$ levels at 200 mM, $p < 0.05$.

than control cells (compare Fig. 10, a and b). Chronic adenosine deaminase treatment (1 unit/ml; 48 hr) did not affect the morphological appearance of the cells (Fig. 10d) and, importantly, adenosine deaminase did not reverse the effect of ethanol on the morphological appearance of NG108-15 cells (Fig. 10c). Furthermore, chronic ethanol treatment markedly inhibited NG108-15 cell division (by 51%), but the cellular protein content was increased by 36% (Fig. 11), indicating larger cells. In additional experiments the inhibitory effect of ethanol on cell division was not blocked by chronic adenosine deaminase treatment, whereas chronic adenosine deaminase treatment alone did not affect cell division, compared with control (data not shown). Ethanol-treated cells were still viable, as assessed by their ability to exclude trypan blue (not shown).

Discussion

Chronic ethanol treatment of NG108-15 cells has been reported to cause a reduction in $G_{s\alpha}$ mRNA, with the subsequent

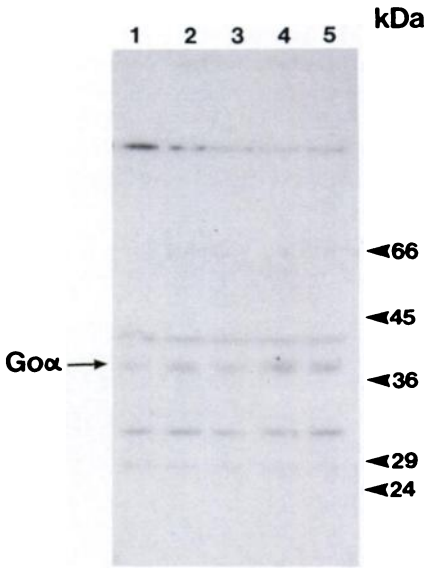

Fig. 4. Concentration-dependent up-regulation of $G_{o\alpha}$ by 48-hr pretreatment with ethanol (10–200 mM). Data are shown from a single experiment. *Lanes* are as in Fig. 2.

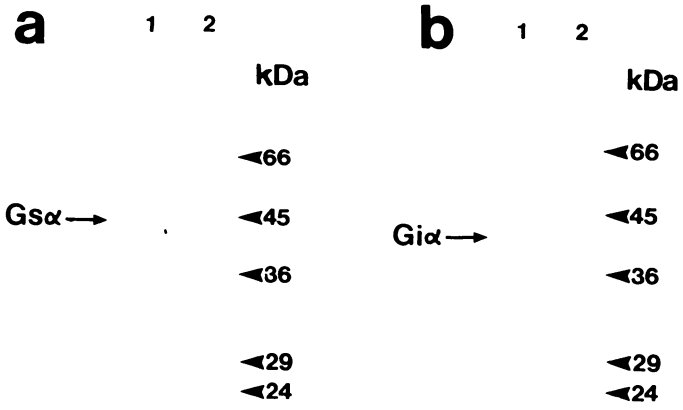

Fig. 5. Effect of 48-hr pretreatment with 200 mM ethanol on the expression of $G_{s\alpha}$ and $G_{i\alpha}$ in membranes of NCB-20 neuroblastoma hybrid cells. Results are shown for $G_{s\alpha}$ (a) and $G_{i\alpha}$ (b) in control (*lanes 1*) and ethanol-pretreated (*lanes 2*) cells.

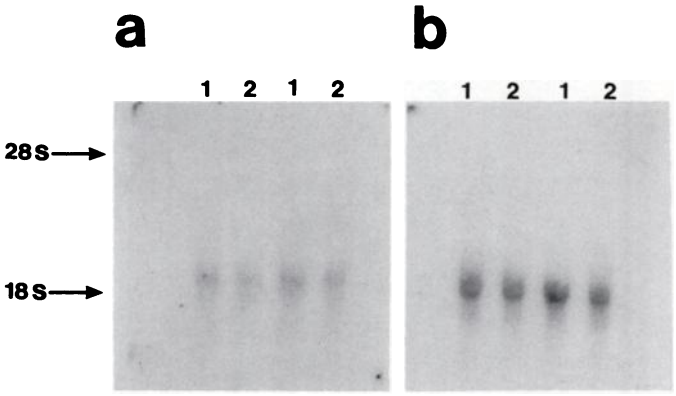

Fig. 6. Northern blot, demonstrating the inability of ethanol pretreatment (200 mM; 48 hr) to reduce $G_{i\alpha 2}$ (a) and $G_{s\alpha}$ (b) mRNA levels in NG108-15 cells. Results are shown for $G_{i\alpha 2}$ mRNA in control (a, *lanes 2*) and ethanol-pretreated (a, *lanes 1*) cells and for $G_{s\alpha}$ mRNA in control (b, *lanes 2*) and ethanol-pretreated (b, *lanes 1*) cells.

loss of membrane $G_{s\alpha}$ protein and the development of heterologous desensitization of prostaglandin E_1- and adenosine receptor-stimulated cAMP production (3, 5). This is proposed to involve ethanol-mediated inhibition of adenosine uptake into the cells (4, 8). Adenosine is released from NG108-15 cells, so that any reduction in the uptake of adenosine would lead to an accumulation of extracellular adenosine, with consequent stimulation of adenosine A_2 receptors present on the cells. It is not known how increased extracellular adenosine leads to heterologous desensitization in these cells, but ethanol itself appears to require the presence of cAMP-dependent protein kinase for the inhibition of adenosine uptake to occur (18).

A difficulty with this hypothesis, however, is that prolonged activation of A_2 receptors on NG108-15 cells has been shown clearly to induce only homologous desensitization of adenylate cyclase activation (9). Furthermore, chronic increases in cAMP levels in NG108-15 or chronic activation of the cAMP-dependent protein kinase leaves G_s function intact (10). Thus, it is not easy to conceive how elevated extracellular adenosine or increased intracellular cAMP leads to heterologous desensitization in these cells. In this study we investigated the effect of chronic ethanol treatment on the expression of different G protein α subunits in NG108-15 cells, reasoning that changes in expression may not be related specifically to G_s. A previous study reported no change in $G_{i\alpha}$ levels (7), but the authors did not investigate $G_{o\alpha}$ expression. We also investigated the effect of chronic ethanol treatment on cAMP signal transduction, in an attempt to correlate changes in G protein α subunit expression with possible changes in this second messenger system. The experimental parameters we used (length of ethanol treatment and ethanol concentrations) were similar to those described previously (3–5).

Our initial results indicated that chronic ethanol treatment dramatically alters the expression of G protein α subunits in membranes of NG108-15 cells. $G_{s\alpha}$ and $G_{i\alpha}$ levels were reduced, whereas $G_{o\alpha}$ levels were increased. In fact, $G_{s\alpha}$ levels were altered less than were levels of the other two α subunits ($G_{s\alpha}$, −24%; $G_{i\alpha}$, −34%; $G_{o\alpha}$, +43%). This indicates that ethanol does not interact selectively with G_s in these cells and also that the effects are unlikely to be due to nonspecific toxic interactions, because in that case we might have expected levels of all three G protein α subunits to decrease. Furthermore, the results in NCB-20 cells indicate that these changes are not unique to the NG108-15 cell line. One possibility to explain the previously reported selective action of ethanol on $G_{s\alpha}$ (7) is that the effect of ethanol on $G_{s\alpha}$ is more potent than that on $G_{i\alpha}$; however, ethanol concentration-effect experiments established that the reductions of $G_{s\alpha}$ and $G_{i\alpha}$ proteins occur at broadly similar ethanol concentrations. It should be noted that the $G_{i\alpha}$ antibody we used recognizes $G_{i\alpha 1}$ and $G_{i\alpha 2}$ but not $G_{i\alpha 3}$. NG108-15 cells do not contain detectable levels of $G_{i\alpha 1}$ (19), so that the band we detect at 40 kDa corresponds only to $G_{i\alpha 2}$ and not to $G_{i\alpha 3}$.

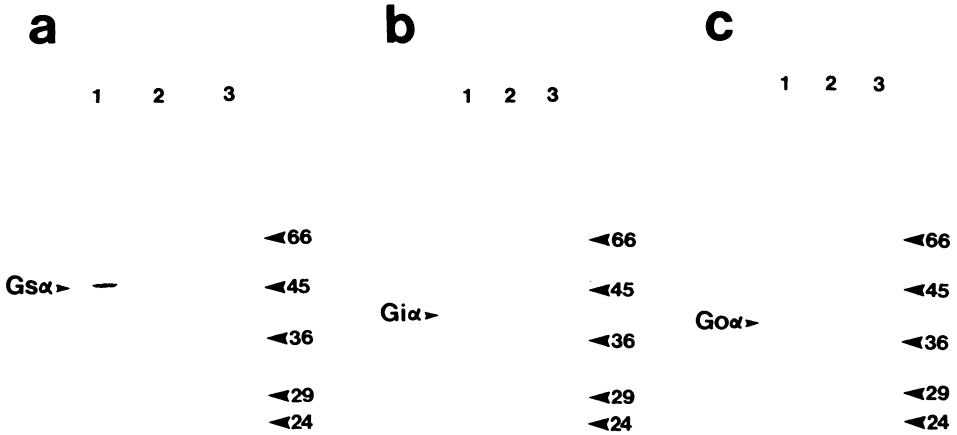

Fig. 7. Western blots, demonstrating the inability of coincubation with adenosine deaminase (1 unit/ml; 48 hr) to reverse the changes in α subunit expression produced by ethanol (200 mM; 48 hr) in NG108-15 cell membranes. Results are shown for $G_{s\alpha}$ (a), $G_{i\alpha}$ (b), and $G_{o\alpha}$ (c), for controls (*lanes 1*), ethanol treatment alone (*lanes 2*), and ethanol plus adenosine deaminase (*lanes 3*).

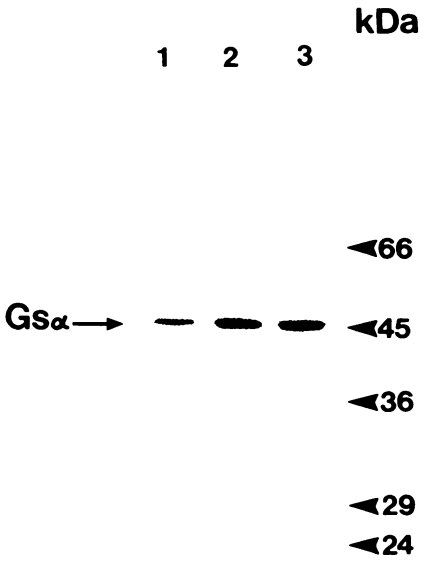

Fig. 8. Inability of NECA pretreatment (10 μM; 48 hr) to mimic the effect of ethanol (200 mM; 48 hr) on $G_{s\alpha}$ expression in NG108-15 cell membranes. Cells were given either no pretreatment (control; *lane 2*), pretreatment with 200 mM ethanol (*lane 1*), or pretreatment with 10 μM NECA (*lane 3*).

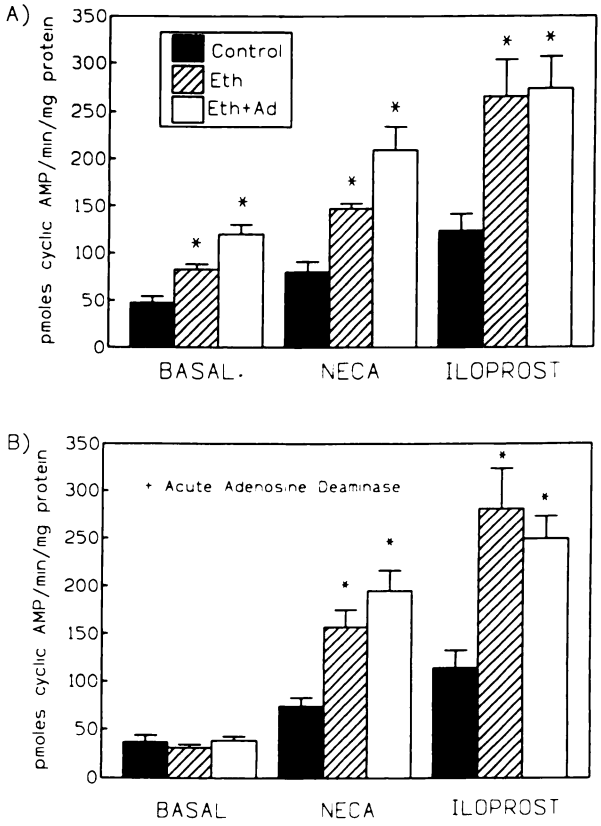

Fig. 9. Effect of chronic treatments on basal and agonist-stimulated cAMP formation in NG108-15 cells. A, Control cells (■), cells pretreated with 200 mM ethanol for 48 hr (▨), or cells pretreated with 200 mM ethanol and 1 unit/ml adenosine deaminase for 48 hr (□) were washed and resuspended in Krebs-Ringer-HEPES buffer containing 1 mg/ml bovine serum albumin and 100 μM Ro20 1724 before being challenged with no drug (basal), 10 μM NECA, or 10 μM iloprost for 10 min. B, Results from such an experiment except that adenosine deaminase (1 unit/ml) was included for the 10-min incubation. Values shown are means ± standard errors of three to six separate experiments for each condition. *, Values significantly greater than respective controls, $p < 0.05$.

However, it has been shown that $G_{i\alpha 2}$ mediates inhibition of adenylate cyclase in these cells (20).

It is possible that the changes in α subunit expression are the result of alterations in the steady state levels of α subunit mRNA, resulting from changes in either gene transcription or mRNA stability. To investigate this we extracted mRNA from control and ethanol-pretreated cells and subjected it to Northern analysis using cDNA probes. However, we observed no decrease in the levels of these transcripts after ethanol treatment, suggesting that changes in mRNA levels do not underlie the observed changes in α subunit expression. Our finding is at variance with a previous report (5), which did find a reduction in $G_{s\alpha}$ mRNA. It does, however, remain possible that ethanol induces short-lived changes in α subunit mRNA levels, which return to normal by the time cells are harvested. It should be noted that it is unlikely that the $G_{i\alpha 2}$ probe hybridizes with either $G_{i\alpha 1}$ or $G_{i\alpha 3}$ mRNA in these cells, because as mentioned above these cells do not express $G_{i\alpha 1}$ and so are unlikely to contain significant amounts of this transcript. Furthermore, $G_{i\alpha 3}$ hybridizes to a single band corresponding to a much larger mRNA in these cells,[1] but we observed only a single band of 1.8–1.9 kb with the $G_{i\alpha 2}$ cDNA.

Chronic ethanol treatment has been reported to reduce $G_{s\alpha}$ levels and induce heterologous desensitization in NG108-15 cells (5). Furthermore, the heterologous desensitization was reversed by addition of adenosine deaminase to the medium (4), implying that ethanol-induced increases in extracellular adenosine mediate the desensitization. However, the ability of adenosine deaminase to reverse the effects of ethanol on $G_{s\alpha}$ levels was not investigated in that study. We, therefore, tested the ability of adenosine deaminase to reverse the changes in α subunit expression induced by ethanol. Our results clearly show that adenosine does not mediate the effects of ethanol on the reduction of $G_{s\alpha}$ and $G_{i\alpha}$ and the increase of $G_{o\alpha}$. We further tested this hypothesis by adding an adenosine agonist to the cells for 48 hr, reasoning that if increased extracellular adenosine and intracellular cAMP mediate the changes in α subunit expression then chronic treatment with an adenosine agonist should mimic these effects. However, chronic NECA treatment did not alter membrane levels of the α subunits. This indicates that, although adenosine may mediate some effects of ethanol in relation to adenylate cyclase activity (4), it does not mediate the changes in α subunit expression.

In contrast to earlier reports (3, 4), we observed that chronic ethanol treatment of NG108-15 cells did not induce heterologous desensitization of agonist-stimulated cAMP formation. Rather, in our hands chronic ethanol treatment enhanced agonist-induced cAMP formation. Furthermore, because chronic inclusion of adenosine deaminase did not reverse the increase in agonist-stimulated cAMP formation due to ethanol,

[1] R. J. Williams, M. A. Veale, P. Horne, and E. Kelly, unpublished observations.

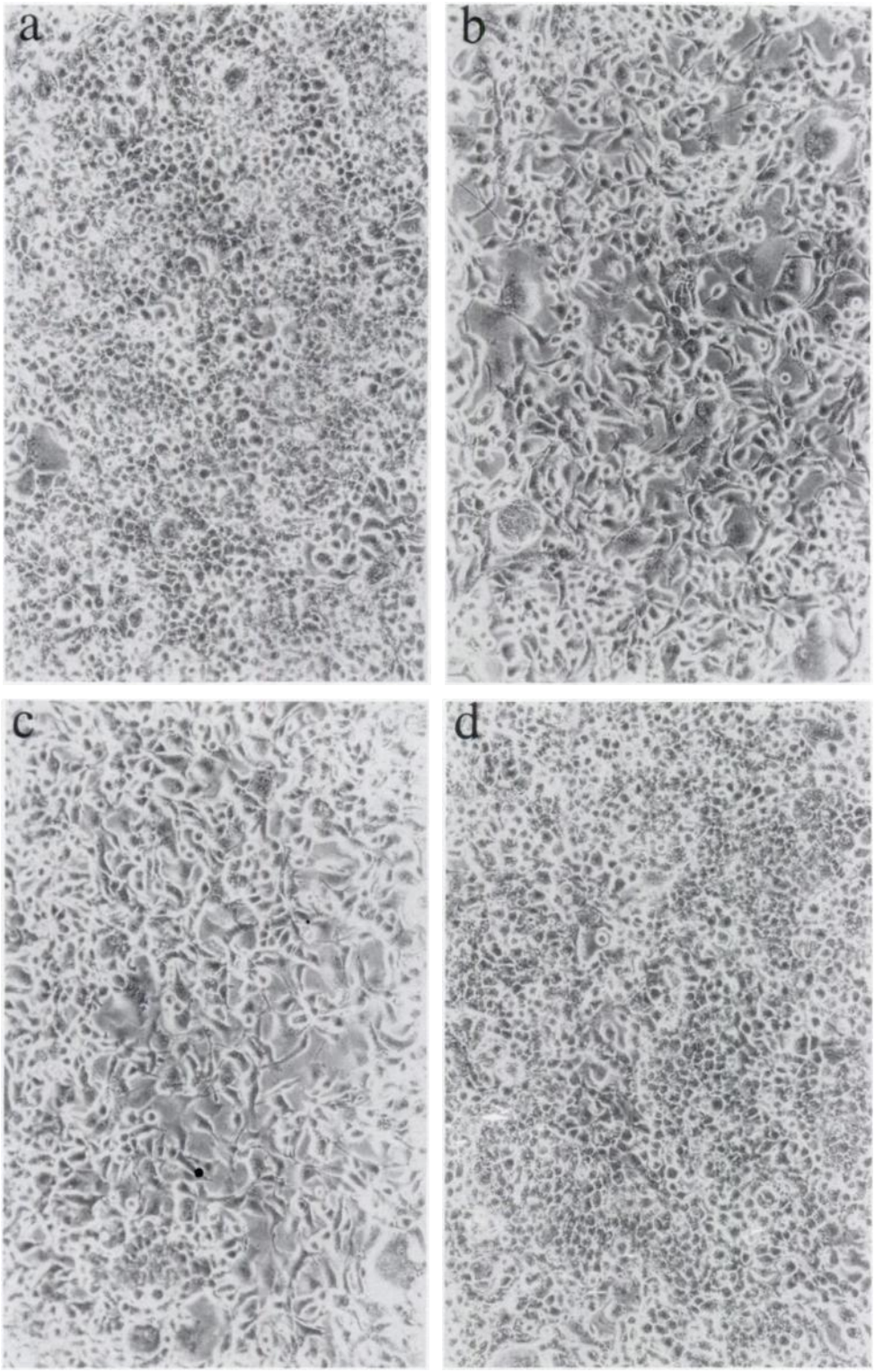

Fig. 10. Morphological appearance of NG108-15 cells with no pretreatment (a), ethanol pretreatment (200 mM; 48 hr) (b), ethanol plus adenosine deaminase pretreatment (1 unit/ml; 48 hr) (c), or adenosine deaminase pretreatment alone (d).

adenosine cannot mediate this effect. In addition, changes in $G_{s\alpha}$ and $G_{i\alpha}$ levels do not provide an obvious explanation for the increase in agonist-stimulated cAMP formation, because $G_{s\alpha}$ levels were reduced, and we might expect the functional consequences of reduced $G_{i\alpha}$ to be cancelled out by the reduction in $G_{s\alpha}$. Interestingly, chronic ethanol treatment has recently been reported to enhance agonist-stimulated cAMP accumulation in cultured hepatocytes (21), the mechanism of which appears to be in part a reduction in $G_{i\alpha}$ expression. In our case it appears more likely that the changes we have observed in agonist-stimulated cAMP accumulation are due to changes in receptor number or to the adenylate cyclase enzyme itself. In this respect, chronic ethanol treatment is known to alter receptor number, as reported for prostaglandin E_1 receptors in N1E-115 cells (2) and opiate receptors in NG108-15 cells (22). Chronic ethanol treatment increased basal cAMP accumulation

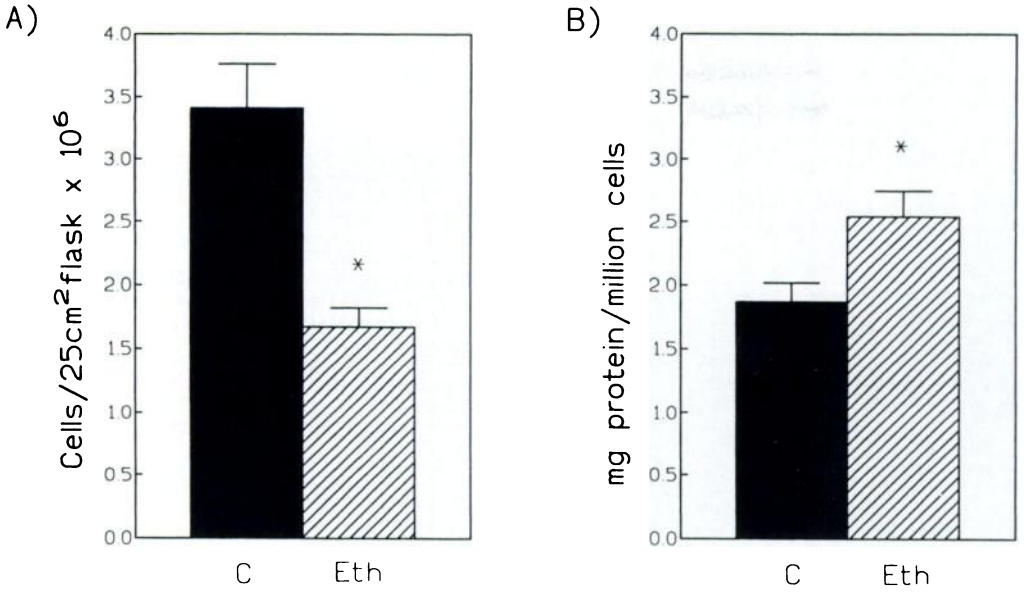

Fig. 11. Effect of chronic ethanol (200 mM; 48 hr) on cell number (A) and protein/cell (B) for NG108-15 cells. Four flasks of control cells (C) and four of ethanol-treated cells (*Eth*) were harvested in phosphate-buffered saline, and samples were taken for cell counting in a hemocytometer or for protein assay. Values are means ± standard errors. *, Ethanol-treated significantly different from control, $p < 0.05$.

in NG108-15 cells. This was due to the increased synthesis and/or release and/or reduced uptake of adenosine, because it was abolished by acute adenosine deaminase treatment. Surprisingly, chronic adenosine deaminase treatment enhanced the chronic ethanol treatment-induced increase in basal cAMP accumulation, and this was also abolished by acute adenosine deaminase treatment. It is unclear at present why chronic ethanol treatment induces this effect; ethanol has been reported to inhibit adenosine uptake in NG108-15 cells (8), but under our experimental conditions to measure cAMP accumulation there is presumably no ethanol left in contact with the cells. It is possible that the increase in basal cAMP that we see is a rebound effect, resulting from the withdrawal of ethanol from the cells. It is unclear why the present results are at variance with previous studies (3, 4, 7). One possibility is that, whereas we used fetal calf serum in our cell cultures, other studies have used defined medium.

During the course of these experiments it became clear that chronic ethanol treatment induced morphological changes in NG108-15 cells. Ethanol inhibited cell division and the cells began to take on a more differentiated appearance, becoming larger and possessing more neurite-like processes than untreated cells. In terms of G protein α subunit levels, these results imply that the number of $G_{s\alpha}$ and $G_{i\alpha}$ subunits per cell may not change but they are consequently more dilute in the cell membrane than in untreated cells. For example, 200 mM ethanol reduced $G_{i\alpha 2}$ levels per unit of protein by 34%, but because there was approximately 36% more protein per cell in ethanol-treated cultures the number of $G_{i\alpha 2}$ subunits per cell did not change significantly. Conversely, the increase in $G_{o\alpha}$ subunits per cell was much greater than the 43% increase per unit of protein shown in Fig. 1. Given the ethanol-induced changes in NG108-15 cell morphology, it is possible that the changes in α subunit expression are the result of cell differentiation. This is supported by a study in which differentiation of NG108-15 cells by chronic enhancement of cAMP levels led to the same reciprocal changes in $G_{i\alpha}$ and $G_{o\alpha}$ that we have observed (23). However, in that study levels of $G_{s\alpha}$, as assessed by cholera toxin-induced ADP-ribosylation, were unchanged by cAMP-mediated differentiation. Chronic ethanol treatment has recently been reported to increase protein kinase C activity in cultured NG108-15 cells (12). Because activators of protein kinase C can be used to differentiate various cell types (24), it is possible that ethanol-induced increases in protein kinase C activity initiate differentiation of the cells, as observed in our experiments. In relation to this, chronic ethanol treatment has been reported to increase the number of voltage-sensitive Ca^{2+} channels in PC12 cells (25). Because these Ca^{2+} channels are probably associated with the $G_{o\alpha}$ protein (26), this could explain the marked increase in $G_{o\alpha}$ levels observed in the present study. Furthermore, G_o is a major growth cone protein (27) and, therefore, would be expected to increase during neuronal differentiation and neurite outgrowth.

In summary, we find that chronic ethanol treatment induces marked changes in NG108-15 cell G protein α subunit expression and cAMP signal transduction. These changes appear unlikely to be due to altered mRNA levels and do not occur as a result of changes in extracellular adenosine concentration. Our results argue against a selective interaction of ethanol with the G_s signal-transducing protein. Instead, we conclude that the changes in α subunit expression are more likely to occur subsequent to a complex interplay of ethanol-induced mechanisms, such as inhibition of cell division, increased cell differentiation, or changes in the expression of proteins involved in protein sorting and trafficking, such as the Hsc70 heat shock protein (11).

References

1. Hoffman, P. L., and B. Tabakoff. Ethanol and guanine nucleotide binding proteins: a selective interaction. *FASEB J.* **4**:2612-2622 (1990).
2. Richelson, E., S. Stenstrom, C. Forray, L. Enloe, and M. Pfenning. Effects of chronic exposure to ethanol on the prostaglandin E_1 receptor-mediated response and binding in a murine neuroblastoma clone (NIE-115). *J. Pharmacol. Exp. Ther.* **239**:687-692 (1986).
3. Gordon, A. S., K. Collier, and I. Diamond. Ethanol regulation of adenosine-receptor stimulated cAMP levels in a clonal neural cell line: an *in vitro* model of cellular tolerance to ethanol. *Proc. Natl. Acad. Sci. USA* **83**:2105-2108 (1986).
4. Nagy, L. E., I. Diamond, K. Collier, L. Lopez, B. Ullman, and A. S. Gordon. Adenosine is required for ethanol-induced heterologous desensitization. *Mol. Pharmacol.* **36**:744-748 (1989).
5. Mochly-Rosen, D., F. Chang, L. Cheever, M. Kim, I. Diamond, and A. S. Gordon. Chronic ethanol causes heterologous desensitization of receptors by reducing α_s mRNA. *Nature (Lond.)* **333**:848-850 (1988).
6. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta* **1031**:163-224 (1990).

7. Charness, M. E., L. A. Querimit, and M. Henteleff. Ethanol differentially regulates G proteins in neural cells. *Biochem. Biophys. Res. Commun.* **155:**138–143 (1988).
8. Nagy, L. E., I. Diamond, D. J. Casso, C. Franklin, and A. S. Gordon. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J. Biol. Chem.* **265:**1946–1951 (1990).
9. Keen, M., E. Kelly, P. Nobbs, and J. MacDermot. A selective binding site for ^{3}H-NECA that is not an adenosine receptor. *Biochem. Pharmacol.* **38:**3827–3833 (1989).
10. Keen, M., E. Kelly, A. Krane, A. Austin, R. Wiltshire, N. Taylor, K. Docherty, and J. MacDermot. Cyclic AMP produces desensitization of prostacyclin and adenosine A_2 receptors in hybrid cell lines but does not affect G_s function. *Biochim. Biophys. Acta* **1134:**157–163 (1992).
11. Miles, M. F., J. E. Diaz, and V. S. DeGuzman. Mechanisms of neuronal adaptation to ethanol: ethanol induces Hsc 70 gene transcription in NG108-15 neuroblastoma × glioma cells. *J. Biol. Chem.* **266:**2409–2414 (1991).
12. Messing, R. O., P. J. Petersen, and C. J. Henrich. Chronic ethanol exposure increases levels of protein kinase C δ and ε and protein kinase C-mediated phosphorylation in cultured neural cells. *J. Biol. Chem.* **266:**23428–23432 (1991).
13. Gayer, G. G., A. Gordon, and M. F. Miles. Ethanol increases tyrosine hydroxylase gene expression in N1E-115 neuroblastoma cells. *J. Biol. Chem.* **266:**22279–22284 (1991).
14. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
15. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227:**680–685 (1970).
16. Stallcup, M. R., and L. D. Washington. Region-specific initiation of mouse mammary tumor virus RNA synthesis by endogenous RNA polymerase II in preparations of cell nuclei. *J. Biol. Chem.* **258:**2802–2807 (1983).
17. Brown, B. L., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121:**561–562 (1971).
18. Nagy, L. E., I. Diamond, and A. S. Gordon. cAMP-dependent protein kinase regulates inhibition of adenosine transport by ethanol. *Mol. Pharmacol.* **40:**812–817 (1991).
19. Milligan, G., F. R. McKenzie, S. J. McClue, F. M. Mitchell, and I. Mullaney. Guanine nucleotide binding proteins in neuroblastoma × glioma hybrid NG108-15 cells: regulation of expression and function. *Int. J. Biochem.* **22:**701–707 (1990).
20. McKenzie, F. R., and G. Milligan. δ-Opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine nucleotide binding protein G_{i2}. *Biochem. J.* **267:**391–398 (1990).
21. Nagy, L. E., and E. F. DeSilva. Ethanol increases receptor-dependent cyclic AMP production in cultured hepatocytes by decreasing G_i-mediated inhibition. *Biochem. J.* **286:**681–686 (1992).
22. Charness, M. E., L. A. Querimit, and I. Diamond. Ethanol increases the expression of functional *delta*-opioid receptors in neuroblastoma × glioma NG108-15 hybrid cells. *J. Biol. Chem.* **261:**3164–3169 (1986).
23. Mullaney, I., A. I. Magee, C. G. Unson, and G. Milligan. Differential regulation of amounts of the guanine nucleotide binding proteins G_i and G_o in neuroblastoma × glioma hybrid cells in response to dibutyryl cyclic AMP. *Biochem. J.* **256:**649–656 (1988).
24. Nishizuka, Y. Studies and perspectives of protein kinase C. *Science (Washington D. C.)* **233:**305–312 (1986).
25. Messing, R. O., C. L. Carpenter, I. Diamond, and D. A. Greenberg. Ethanol regulates calcium channels in clonal neural cells. *Proc. Natl. Acad. Sci. USA* **83:**6213–6215 (1986).
26. Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz. The GTP-binding protein, G_o regulates neuronal calcium channels. *Nature (Lond.)* **325:**445–447 (1986).
27. Strittmatter, S. M., D. Valenzuela, T. E. Kennedy, E. J. Neer, and M. C. Fishman. G_o is a major growth cone protein subject to regulation by GAP-43. *Nature (Lond.)* **344:**836–841 (1990).

Send reprint requests to: Eamonn Kelly, Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK.