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ETHANOL DIFFERENTIALLY REGULATES G PROTEINS IN NEURAL CELLS

Michael E. Charness, Lisa A. Querimit, and Mark Henteleff

Department of Neurology and the Gallo Center, University of California, San Francisco, Building 1, Room 101, San Francisco General Hospital, San Francisco, CA 94110

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SUMMARY: Long-term incubation of clonal neural cell lines with ethanol differentially reduces the stimulation of cAMP accumulation by hormones and cholera toxin. In the NG108-15 neuroblastoma x glioma hybrid cell line, this heterologous desensitization was associated with a 42% reduction in the expression of $G_s\alpha$ and no significant change in $G_i\alpha$. By contrast, ethanol treatment of the parental neuroblastoma cell line N18TG2 caused little loss of response to hormones or cholera toxin and no significant change in $G_s\alpha$ or $G_i\alpha$. Ethanol induced heterologous desensitization in N1E-115 neuroblastoma cells; however, this cell line showed a dose-dependent increase in $G_i\alpha$ and a later decrease in $G_s\alpha$. Thus, ethanol causes heterologous desensitization of hormone-stimulated cAMP accumulation by different mechanisms in related neural cell lines.

There is increasing evidence that adaptive responses to ethanol, such as tolerance and physical dependence, may be mediated, in part, by changes in the hormonal regulation of adenylyl cyclase (E.C.4.6.1.1). Cerebral cortical membranes from ethanol-dependent mice show diminished high-affinity agonist binding to the β-adrenergic receptor (1) and diminished stimulation of adenylyl cyclase by isoproterenol and guanine nucleotides (2). Similarly, prior treatment of cultured neural cells with ethanol reduces the stimulation of cAMP accumulation by prostaglandin E_1 (PGE_1), phenylisopropyladenosine (PIA), and cholera toxin (CTX) (3-5). Lymphocytes and platelets from alcoholic patients also show reduced hormonal stimulation of cAMP accumulation and adenylyl cyclase activity, respectively (6,7). While the mechanism by which ethanol produces these effects is unknown, the observation of heterologous loss of hormone stimulation, diminished responses to cholera toxin and guanine nucleotides, and loss of high-affinity agonist binding all suggest changes in the pathway of signal transduction distal to the hormone receptor.

Adenylyl cyclase activity is coordinately regulated by stimulatory and inhibitory hormones acting respectively through G_s and G_i, the stimulatory and inhibitory GTP-binding regulatory proteins (8). Prolonged exposure to hormones or other agents may produce adaptive decreases in hormonal responses. Heterologous desensitization, the loss of responsiveness to more than one class of hormones, occurs in different tissues through a variety of mechanisms

Abbreviations: PGE_1, prostaglandin E_1; PIA, phenylisopropyladenosine, CTX, cholera toxin.

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including heterologous modification of receptors (9) and changes in the abundance or function of G_s and G_i (10,11).

Physiological and biochemical responses to ethanol differ among diverse neurons (12), brain regions (13,14), and individuals (15). This variability of responsiveness is also evident in clonal neural cell lines derived from the C1300 mouse neuroblastoma, which show varying degrees of heterologous desensitization following chronic ethanol exposure (5). Here we use specific antisera (16) to demonstrate that chronic treatment of these cell lines with ethanol differentially regulates the expression of the α-subunits of G_s and G_i.

MATERIALS AND METHODS

Cell Culture All cell lines were cultured in T175 flasks (Falcon) containing Dulbecco's modified Eagle's medium (DMEM) (DMEH-21, Grand Island Biological Company) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.1 mM hypoxanthine/1 μM aminopterin/12 μM thymidine (NG108-15) or 100 μM thioguanine (N18TG2), as described previously (17).

Cyclic AMP Measurements Triplicate samples of confluent cells attached to 4.5 cm^2 multiwell trays were preincubated for 30 min at 37°C in DMEM containing 25 mM HEPES (pH 7.4), 2 mM glutamine, 100μM of the phosphodiesterase inhibitor 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62,711; Schering AG), and 1 U/ml adenosine deaminase (Buffer A)(5). Cells were then incubated for 15 minutes at 37°C in 0.5 ml of buffer A containing 100 μM PIA, a maximally effective concentration. For stimulation of cAMP accumulation by CTX, cells were preincubated for 1 h at 37°C in 0.5 ml buffer A containing 100 nM CTX, the media were changed, and cells were incubated a further 30 min in new CTX-containing buffer. Cyclic AMP was extracted and measured as described previously (17).

Membrane Preparation Washed cells ($20x10^6$) were suspended in 10 ml of lysis buffer (5 mM $MgCl_2$, 1 mM EGTA, 25 mM Tris-HCl, pH 7.4 at 4°C), placed on ice for 10 min, and homogenized with 10 strokes of a teflon-glass homogenizer. The nuclear pellet was removed by centrifugation at 500 x g for 10 min, and the supernatant was centrifuged at 40,000 x g for 20 min at 4°C. The membrane pellet was washed twice by centrifugation and resuspended in assay buffer by 10 passes through a 26 gauge needle. Membranes were frozen at -70°C and used for immunoblots within 6 weeks.

Antibodies Antisera 572 and 584 were generous gifts of Drs. Susanne Mumby and Alfred Gilman, University Texas, Dallas, and antiserum 569 was provided by Dr. Ryn Miake-Lye, California Institute of Technology. Antiserum 584 was generated to a synthetic peptide CTPEPGEDPRVTRAKY derived from the cloned sequence of $G_s\alpha$ and, like antiserum 572 (16), is highly selective for $G_s\alpha$ (Dr. Alfred Gilman, personal communication). Antiserum 569 selectively recognizes $G_i\alpha$ and $G_o\alpha$ (16).

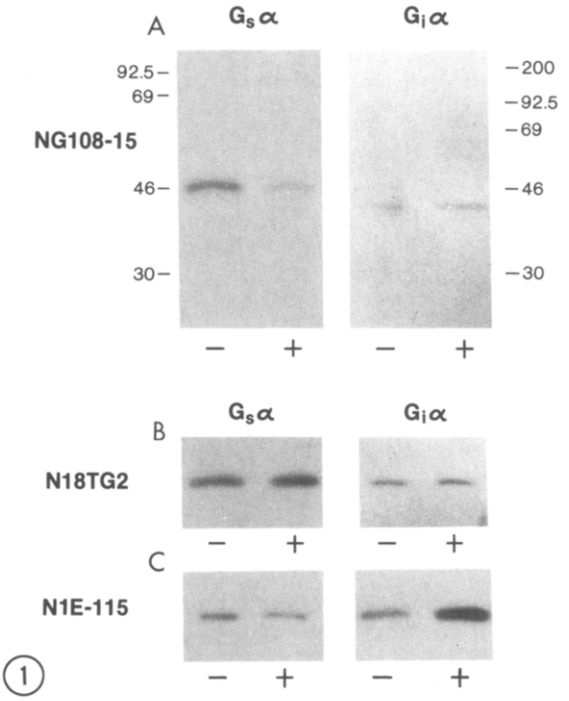
Western Blots Membrane proteins (30 μg) were separated by SDS-PAGE (1.5 mm thick, 10% acrylamide) using the Laemmli buffer system (18), and electrophoretically transferred to 0.2 μm nitrocellulose filters. The filters were rinsed and then incubated successively for 2 h at room temperature in 15 ml of the following solutions: 1. blot buffer (3% bovine serum albumin, 0.3% Tween 20, and 0.02% Na azide dissolved in phosphate-buffered saline, pH 7.4); 2. blot buffer + antiserum (dilutions: 572, 1:500; 584, 1:1000; 569, 1:2000); and 3. ^{125}I-protein A (Amersham, 30 mCi/mg; 1 μCi/15 ml). Unbound antibody was removed by 5x5 minute washes in phosphate-buffered saline containing 0.3% Tween 20 (pH 7.4) and saved for 2 additional immunoblots. Unbound ^{125}I-protein A was removed similarly, except that the wash buffer was supplemented with 0.5 M NaCl. Dried filters were placed for autoradiography at -70°C (3 days for $G_i\alpha$, 1 day for $G_s\alpha$). Bands from control and ethanol-treated cells were compared by quantitative densitometry.

Statistical Analysis Data are expressed as mean$\pm$S.E.. Means were compared using the two-tailed Student's t test for paired samples with statistical significance defined as $p<.05$.

RESULTS AND DISCUSSION

In all cell lines, antisera 572 and 584 bound to a single band with a relative molecular mass of 46 kDa, consistent with $G_s\alpha$ (16). Western blots with antiserum 569 showed a major band at approximately 41 kDa and an occasional minor band at 39 kDa, consistent with $G_i\alpha$ and $G_o\alpha$, respectively (16). The band stained by antiserum 572 or 584 will be referred to as $G_s\alpha$ and the major band stained by antiserum 569 as $G_i\alpha$.

NG108-15 is a hybrid cell line derived from the fusion of the mouse neuroblastoma N18TG2 and rat glioma C6BU-1 cell lines (19). Prior incubation of NG108-15 cells with 200 mM ethanol for 48 h reduced the accumulation of cAMP stimulated by PGE_1 (20% reduction), PIA (11%), and CTX (30%), but not forskolin (5). Western blots of membranes prepared from ethanol-treated NG108-15 cells showed a 42±6% reduction in $G_s\alpha$ (n=6, p<.001)(Figures 1a and 2). There was no significant change in $G_i\alpha$, arguing against a non-specific effect of ethanol on the expression of the α-subunits of G proteins.



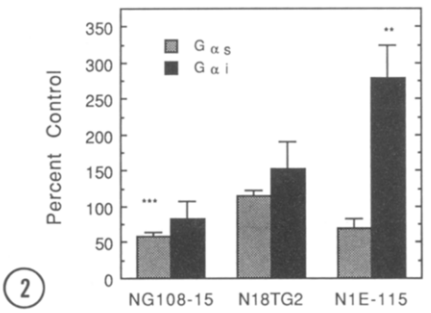


Figure 1. *Western blot analysis of $G_s\alpha$ and $G_i\alpha$ in ethanol-treated clonal neural cell lines.* Cells were incubated for 48 h in the absence (-) or presence (+) of 200 mM ethanol. Western blots of membrane proteins are shown for representative experiments using NG108-15 cells (A), N18TG2 cells (B), and N1E-115 cells (C).

Figure 2. *Quantitative analysis of ethanol-induced changes in $G_s\alpha$ and $G_i\alpha$.* Autoradiograms from the experiments of the type shown in Figure 1 were analyzed by quantitative densitometry using multiple film exposures. In each experiment the density of bands was compared between adjacent lanes containing 30 μg of membrane protein from control and ethanol-treated cells. Each bar shows the mean±SE percent of control density for ethanol-treated NG108-15 (n=5), N18TG2 (n=4) and N1E-115 (n=7) cells.
** p<.01; *** p<.001, relative to control density.

G_s and G_i are heterotrimers consisting of distinct α-subunits and similar $\beta\gamma$ subunits (8). Receptor activation promotes the subunit dissociation of G_s, leading to activation of adenylyl cylase by free $G_s\alpha$. Cholera toxin stimulates cAMP accumulation by blocking the intrinsic GTPase activity that terminates the action of $G_s\alpha$. The loss of $G_s\alpha$ in ethanol-treated NG108-15 cells might therefore be responsible for the roughly equivalent loss of CTX response (5). Additional effects of ethanol on the adenosine A_2 and PGE_1 receptors might account for the smaller reductions in the PIA and PGE_1 responses (5).

The ethanol-induced reduction in $G_s\alpha$ could also conceivably explain why ethanol-treated NG108-15 cells show an increase in maximal opioid inhibition of hormone-stimulated cAMP accumulation (17,21). Opioid and other G_i-coupled receptors promote the subunit dissociation of G_i, releasing $\beta\gamma$ subunits to complex and inactivate $G_s\alpha$ (8). A decrease in G_s relative to G_i could potentiate maximal agonist inhibition by allowing a given amount of $\beta\gamma$ derived from G_i to inactivate a larger proportion of $G_s\alpha$.

If changes in G proteins mediate ethanol-induced heterologous desensitization, then G proteins should be unchanged in ethanol-treated cell lines that do not show heterologous desensitization. In contrast to NG108-15, the N18TG2 neuroblastoma parental cell line showed relatively little decrease in hormonal or cholera toxin responsiveness after chronic ethanol exposure (5). Likewise, ethanol did not cause any significant changes in $G_s\alpha$ or $G_i\alpha$ (Figures 1b and 2). Regulation of $G_s\alpha$ by ethanol in NG108-15 (N18TG2 x C6BU-1) cells may therefore be dependent on a C6BU-1 gene product. Among four related cell lines, N18TG2 also shows the least induction of the δ-opioid receptor by ethanol (20). This cell line may prove useful in evaluating how ethanol provokes cellular adaptation.

Chronic ethanol treatment in a third cell line, the N1E-115 mouse neuroblastoma, reduced the response to PGE_1 (31%) and PIA (35%), but not CTX (5). This lack of change in CTX response suggests that ethanol diminishes hormonal responsiveness by different mechanisms in NG108-15 and N1E-115 cells. Membranes from ethanol-treated N1E-115 cells did show a reduction in $G_s\alpha$ ($31\pm13\%$,n=7,p=.07); however, the more striking effect was a nearly 3-fold increase in $G_i\alpha$ (n=9, $p<.01$) (Figures 1c and 2). Similar increases in $G_i\alpha$ have been shown to mediate heterologous desensitization in glucagon-treated MDCK cells (10).

Because 200 mM ethanol is a concentration achieved only rarely in man (21), we studied the effects of longer treatment with lower concentrations of ethanol. N1E-115 cells were incubated with 0,25,50,100 or 200 mM ethanol for a total of 5 days. Cells attached to multiwell trays were then assayed in the absence of ethanol for the stimulation of cAMP accumulation by PIA and CTX, while parallel cultures were harvested for Western blots.

Incubation of N1E-115 cells with ethanol for 5 days caused a large, dose-dependent decrease in the response to both CTX and PIA as well as an increase in the expression of $G_i\alpha$ (Figure 3). Although $G_i\alpha$ did not increase significantly between 2 and 5 days of treatment with 200 mM ethanol ($2.8\pm.5$-fold vs $3.1\pm.3$-fold; $p>.05$), the extent of desensitization to PIA nearly doubled. This may have been due to a significant decrease in the expression of $G_s\alpha$ seen at the later time point (for 200 mM ethanol: $63\pm10\%$; n=3;

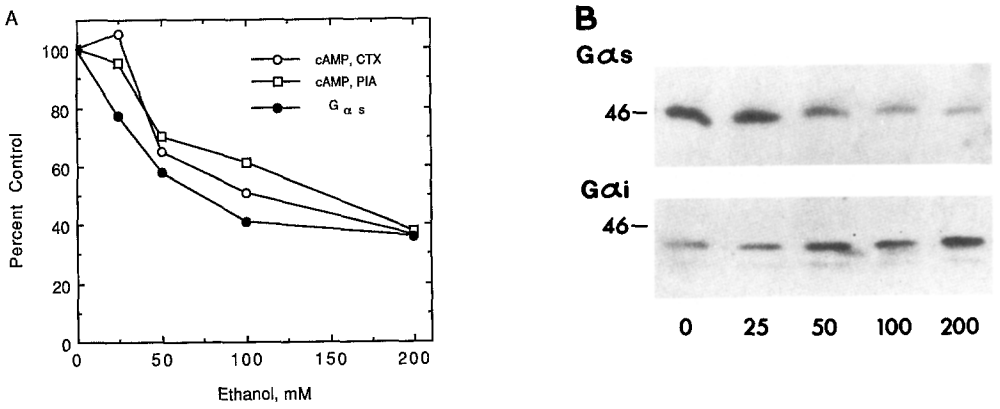


Figure 3. *Dose-response curve for ethanol-induced changes in PIA- and CTX-stimulated cAMP accumulation and expression of $G_s\alpha$ and $G_i\alpha$ in N1E-115 cells.* N1E-115 cells were incubated with 0,25,50,100, or 200 mM ethanol for 5 days. Cyclic AMP accumulation in the absence of ethanol was stimulated by 100 nM CTX or 100 μM PIA. Western blots from the same cells were analyzed for changes in $G_s\alpha$ and $G_i\alpha$. A. Each point represents the mean percentage of the indicated measurement for ethanol-treated compared to control cells from 3 independent experiments. B. Representative Western blots showing the dose-dependent decrease in $G_s\alpha$ and increase in $G_i\alpha$.

$p<.05$)(Figure 3). Indeed, when N1E-115 cells were incubated with 100 mM ethanol for up to 5 days, a gradual, parallel loss of response to PIA and CTX was observed, reaching a maximum after 3 days (Figure 4). Thus, ethanol-induced heterologous desensitization in N1E-115 cells appears to be mediated initially by increases in $G_i\alpha$ and subsequently by additional decreases in $G_s\alpha$.

In these experiments 100 mM ethanol significantly reduced the CTX response after 2 days (by 46±9%, n=4; $p<.02$), whereas previously 200 mM ethanol did not (5). This discrepancy may be related to differences in the sensitivity to ethanol of different cell batches.

The major finding of this study is that ethanol differentially regulates the abundance and function of G proteins in related clonal neural cell lines. In this respect, these cell lines successfully model the diversity of responses to ethanol seen in different neurons (12),

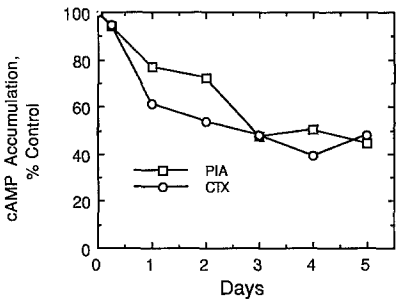


Figure 4. *Time course for the ethanol-induced desensitization of PIA- and CTX-stimulated cAMP accumulation in N1E-115 cells.* N1E-115 cells were incubated with 100 mM ethanol for the indicated time. Each point represents the mean percentage of cAMP accumulation stimulated by 100 nM CTX or 100 μM PIA in ethanol-treated compared to control cells, n=4-5 experiments.

brain regions (13,14), and individuals (15). G proteins mediate transmembrane signaling by numerous neurotransmitters and neuromodulators (8). Ethanol-induced changes in the abundance of G proteins could therefore have far-reaching effects on brain function and might underlie an important element of neural adaptation to ethanol.

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