Ethanol increases receptor-dependent cyclic AMP production in cultured hepatocytes by decreasing G_i -mediated inhibition

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Increasing evidence suggests that ethanol-induced changes in cyclic AMP (cAMP) signal transduction play a critical role in the acute and chronic effects of ethanol. Here we have investigated the effects of ethanol on cAMP signal transduction in primary cultures of rat hepatocytes. Acute exposure to ethanol had a biphasic effect on glucagon-receptor-dependent cAMP production in intact cells: 25–50 mM-ethanol decreased cAMP, whereas treatment with 100–200 mM-ethanol increased cAMP. After chronic exposure to 50–200 mM-ethanol for 48 h in culture, glucagon-receptor-dependent cAMP levels were increased, but no change in glucagon receptor number was observed. These effects of ethanol were independent of ethanol oxidation. Chronic ethanol treatment also increased adenosine-receptor- and forskolin-stimulated cAMP production. Increased cAMP production was also observed upon stimulation of adenylate cyclase with glucagon, forskolin and F^- in membranes isolated from cells cultured with 100 mM-ethanol for 48 h. However, no differences were observed in basal and $MnCl_2$ -stimulated adenylate cyclase activity. The quantity of α_i protein was decreased by 35% after chronic ethanol treatment, but no change in the quantity of α_s protein was detected. Decreased α_i protein was associated with a decrease in G_i function, as assessed by the ability of 0.1 nM-guanosine 5'-[$\beta\gamma$ -imido]triphosphate and 1 μ M-somatostatin to inhibit forskolin-stimulated adenylate cyclase activity. Taken together, these results suggest that chronic exposure to ethanol increases receptor-dependent cAMP production in hepatocytes by decreasing the quantity of α_i protein at the plasma membrane and thereby decreasing the inhibitory effects of G_i on adenylate cyclase activity.

INTRODUCTION

Cyclic AMP (cAMP) is a critical regulator of cellular function [1]. Production of cAMP by adenylate cyclase is co-ordinately regulated by the G-proteins, G_s and G_i , which stimulate and inhibit cAMP production respectively [2]. Several forms of the α -subunits of both G_s and G_i have been identified [3]. Isoform distribution is known to vary between different cell types [3], yet the functional significance of these different isoforms is not well established. G-protein activity can be modulated by a variety of covalent modifications [4–6], as well as by changes in protein quantity [7–11]. Such changes in G-protein activity regulate cAMP production under a variety of physiological conditions such as diabetes [6,9], agonist-induced desensitization (see [12]) and changes in thyroid status [13–15].

Increasing evidence suggests that ethanol-induced changes in cAMP signal transduction may mediate cellular responses to ethanol [16]. In cultured NG108-15 neuroblastoma $\times$ glioma cells and S49 lymphoma cells, acute exposure to ethanol stimulates receptor-dependent cAMP production, whereas long-term treatment decreases cAMP production via receptors positively coupled to adenylate cyclase (heterologous desensitization) [10,17,18]. Ethanol-induced desensitization in NG108-15 cells is correlated with a decrease in mRNA for the α -subunit of G_s (α_s) and a consequent decrease in α_s -subunit protein [10]. However, the interactions of ethanol with receptor-stimulated cAMP production can vary among cell types. For example, acute effects of ethanol on adenylate cyclase activity in PC12 cells vary between clonally derived cell lines [19]. Similarly, chronic ethanol-induced changes in the quantities of α_s and α_i (α -subunit of G_i) vary among different cultured neural cell lines [11]. This diversity of cellular response to both acute and chronic ethanol treatment may contribute to the pleiotropic effects of ethanol, not only on

different cell types within the same tissue, but on different tissues in the whole animal.

Acute and chronic exposure to ethanol alters hepatic metabolism, with the eventual appearance of alcoholic liver disease in 20% of all alcoholics [20,21]. The mechanisms for ethanol-induced changes in hepatocellular function are not well understood. Since cAMP is an important regulator of hepatic metabolism, ethanol-induced changes in cAMP production could contribute to the effects of ethanol on hepatic function. Here we have used primary cultures of rat hepatocytes, which retain significant activity of alcohol dehydrogenase for several days in culture, to investigate the acute and chronic effects of ethanol on receptor-dependent cAMP production.

MATERIALS AND METHODS

Materials

RO 20-1724 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Cell culture reagents were purchased from Grand Island Biological Co. and Flow Laboratories. Antibody to cAMP, ^{125}I -cAMP and ^{125}I -labelled anti-rabbit IgG were purchased from ICN. ^{125}I -glucagon was purchased from Amersham. Antibodies to α_s (GA/1) and α_i (AS/7) were purchased from New England Nuclear. All other reagent-grade chemicals were purchased from Sigma and Boehringer Mannheim.

Hepatocyte isolation and culture

Hepatocytes were isolated by the method of Seglen [22]. Briefly, rats were anaesthetized with sodium pentobarbital and livers were perfused via the portal vein at a flow rate of 15 ml/min, first with modified Hanks' solution (free of Ca^{2+} and Mg^{2+}) containing 1 mM-EGTA and 10 mM-Hepes and then with 0.05%

Abbreviations used: cAMP, cyclic AMP; G_s , stimulatory G-protein; G_i , inhibitory G-protein; NECA, 5'-(*N*-ethylcarboxamido)adenosine; PBS, Dulbecco's phosphate-buffered saline; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]phosphate.

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collagenase (Type I) in Williams E medium with 10 mM-Hepes. A cell suspension was formed by gentle disruption of the collagenase-treated livers in Williams E medium containing 7.5% (v/v) fetal-bovine serum.

Cells were plated at 2×10^5 cells/ml in collagen-coated six-well plates or T-75 culture flasks. After 2 h at 37 °C, non-attached cells were removed by aspiration and the serum-containing media were replaced with Williams E media supplemented with 2 mM-glutamine, 50 units of penicillin/streptomycin/ml, 25 mM-Hepes, 6.3 μ g of insulin/ml, 0.35 mM-L-proline, 10 mM-sodium pyruvate, 50 ng of epidermal growth factor/ml, trace elements and 5 μ g of linoleic acid/ml complexed to fatty-acid free BSA [23,24]. For chronic treatment with ethanol, 0–200 mM-ethanol was added to the cell culture media after 24 h in culture, and plates were wrapped in Parafilm to prevent evaporation of ethanol. Ethanol concentrations in cell culture media were measured by g.l.c. using a Supelco 80/100 Carbowax C/0.2% Carbowax 1500 column. After 48 h in culture, 20 ± 5 mM (mean $\pm$ S.E.M., $n = 5$) of the 100 mM-ethanol added to cell cultures was lost by evaporation and metabolism. Cell number and viability were monitored by cell counting and Trypan Blue exclusion, and did not differ between control and ethanol-treated cells (results not shown).

Receptor-dependent cAMP production in intact cells

After culture in 0–200 mM-ethanol for 48 h in six-well plates, media were removed by aspiration and cells washed once with 2 ml of Dulbecco's phosphate-buffered saline (PBS) with 25 mM-Hepes and 10 mM-glucose. Cells were then pre-treated for 10 min at 37 °C with 10 μ M-RO 20-1724, an inhibitor of phosphodiesterase, and 1 unit of adenosine deaminase/ml, to degrade any endogenously produced adenosine [17]. Varying concentrations of agonist with or without ethanol were then added and incubations continued for 10 min. Reactions were terminated by addition of 100 μ l of 1 M-HCl and 2% Nonidet P-40 and placed on ice for 30 min. Samples were stored at –20 °C and cAMP was determined by radioimmunoassay [17].

cAMP production in isolated membranes

After culture in 0–200 mM-ethanol for 48 h in T-75 flasks, cells were washed with PBS and scraped into 2 ml of ice-cold homogenizing buffer, containing 25 mM sodium phosphate (pH 7.4), 100 mM NaCl, 5 mM-EDTA, 1 mM-EGTA, 10 μ g of leupeptin/ml, 17.5 μ g of aprotinin/ml and 2 mM-MgCl₂. Cells were then homogenized by 15 strokes in a Teflon/glass homogenizer. Unbroken cells and nuclei were separated by centrifugation at 400 *g* for 5 min at 4 °C, and the resulting supernatant was then centrifuged at 100 000 *g* for 30 min at 4 °C. The pellet was resuspended at 0.3 mg of protein/ml in 12.5 mM-Tris/HCl, pH 7.4, with proteinase inhibitors as above. Membranes (30 μ g of protein) were then added to 100 μ l of 12.5 mM-Tris/HCl, pH 8, containing (final concns.) 2 mM-MgCl₂, 10 mM-phosphocreatine, 1 unit of adenosine deaminase/ml, 10 μ M-RO 20-1724, 0.3 mM-ATP, 1 mg of creatine kinase/ml and 1.0 mg of BSA/ml. cAMP produced over 10 min was determined by acetylated radioimmunoassay [17]. cAMP production was linear at this time and protein concentration.

Western blots

Membranes were isolated as described above and resuspended at 1 mg of protein/ml: 25 μ g (for detection of α_1) and 100 μ g (for detection of α_s) of membrane proteins were separated by SDS/PAGE in the Laemmli buffer system [25]. Proteins were then electrophoretically transferred to nitrocellulose membranes [26]. The membranes were blocked with 5% (w/v) non-fat dry milk in 10 mM-Tris/HCl/0.9% NaCl/0.02% NaN₃ (Tris/azide) buffer,

pH 7.0, for 16 h, washed with 4×20 ml of Tris/azide, and incubated with antisera AS/7 or GA/1 (1:1000 dilution in 5% non-fat dry milk). Membranes were again washed and probed with ¹²⁵I-labelled goat anti-rabbit IgG. Unbound ¹²⁵I-labelled goat anti-rabbit IgG was removed by washing, and membranes were dried and placed at –70 °C for autoradiography. Bands from control and ethanol-treated cells were compared by scanning densitometry. Under these conditions, absorptivity of labelled bands was linear from 10 to 100 μ g of membrane protein for α_1 and 25–150 μ g of membrane protein for α_s (results not shown).

Glucagon-receptor binding

Membranes were prepared as described above from control and ethanol-treated cells. Membranes (50 μ g of protein) were incubated in a final volume of 0.25 ml for 20 min with ¹²⁵I-glucagon (0.3 nM, 2000 Ci/mmol) in 20 mM-Hepes, pH 7.6, containing 2% BSA, and 1 mM-EDTA with or without unlabelled glucagon (0.1–10 nM) [27]. Non-specific binding was determined by addition of 3 μ M unlabelled glucagon. The binding reaction was terminated by addition of 3 ml of ice-cold incubation buffer, and bound glucagon was separated by filtration through Whatman GF/C filters [27]. Filters were washed with incubation buffer, dried and counted for radioactivity in a γ -radiation counter. Binding parameters were determined by Scatchard analysis [28].

Alcohol dehydrogenase activity

This was determined in freshly isolated hepatocytes and cells after 48 h in culture by measuring NADH production in the presence of 25 mM-ethanol [29].

Statistical analysis

Values reported are mean $\pm$ S.E.M. Data were analysed by paired *t* test or analysis of variance for repeated measures.

RESULTS

Treatment of primary cultures of rat hepatocytes with ethanol for 10 min (acute exposure) had a biphasic effect on glucagon-receptor-stimulated cAMP production (Fig. 1a). Acute exposure to 25 mM- or 50 mM-ethanol significantly inhibited cAMP production, whereas concentrations greater than 100 mM-ethanol stimulated cAMP production. After chronic exposure to 50–200 mM-ethanol for 48 h, glucagon-receptor-dependent cAMP levels were significantly increased in cultured hepatocytes (Fig. 1b). Challenge of chronically treated cells with acute ethanol still had a biphasic effect on cAMP (Fig. 1a). This chronic ethanol-induced increase in glucagon-receptor-dependent cAMP production in hepatocytes contrasts with decreased receptor-dependent cAMP production observed in a variety of cultured neural cells and lymphocytes after chronic exposure to ethanol [10,16–18].

The first step in stimulation of cAMP production by glucagon is via interaction with its receptor. Therefore increased glucagon-receptor-dependent cAMP production after chronic ethanol treatment might be due to an increase in the quantity of glucagon receptors present on the cell surface. Scatchard analysis of ¹²⁵I-glucagon binding to membranes isolated from control and ethanol-treated hepatocytes showed no difference in $B_{\max}$ for glucagon binding: $B_{\max}$ for control cells was 442 ± 131 fmol/mg of protein, compared with 494 ± 77 ($n = 3$) after culture with ethanol. There was a small increase in K_d after chronic ethanol from 8.9 ± 1.0 to 13.1 ± 0.8 nM (Fig. 2). These results suggest that chronic ethanol acts to increase cAMP production at sites downstream from the glucagon receptor.

Fig. 1. Acute and chronic effects of ethanol on receptor-dependent cAMP production

(a) Cells were cultured with (□) 0 or (■) 100 mM-ethanol for 48 h and then stimulated with 1 μM-glucagon in the presence of 0–200 mM-ethanol for 10 min. Values are expressed relative to cells not treated with acute ethanol (342 ± 63 and 809 ± 30 pmol of cAMP/ 10^6 cells for cells cultured in the absence and presence of ethanol respectively). (b) Cells were cultured with 0–200 mM-ethanol for 48 h and stimulated with 1 μM-glucagon and 1 μM-NECA, an adenosine-receptor agonist. Values are expressed relative to cAMP levels in cells not cultured with ethanol (301 ± 43 pmol/ 10^6 cells for glucagon and 300 ± 44 for NECA). Basal (non-stimulated) levels of cAMP were 60 ± 6 pmol of cAMP/ 10^6 cells in control and 53 ± 6 in cells cultured with 100 mM-ethanol. ($n = 4$, $*P < 0.05$ compared with control.)

Fig. 2. Scatchard analysis of glucagon binding to hepatocytes after chronic exposure to ethanol

125 I-glucagon binding to primary cultures after culture with (○) 0 or (●) 100 mM-ethanol for 48 h. Values are from a representative experiment done in duplicate and replicated three times.

Fig. 3. Acute effects of ethanol on receptor-dependent cAMP production

Hepatocyte cultures were preincubated (□) without and (○) with 10 μM-4-methylpyrazole, an inhibitor of alcohol dehydrogenase, and then stimulated with 1 μM-glucagon in the presence of 0–200 mM-ethanol for 10 min. Values are expressed relative to cAMP levels in cells not treated with ethanol (342 ± 63 and 299 ± 70 pmol of cAMP/ 10^6 cells in the absence and presence of 4-methylpyrazole respectively): $*P < 0.05$ compared with cells not treated with ethanol.

If ethanol were affecting post-receptor component(s) of the cAMP signal-transduction pathway, then chronic exposure to ethanol would be expected to increase cAMP upon stimulation with a variety of receptor agonists. At 1 μM, 5'-(*N*-ethyl-carboxamido)adenosine (NECA), an adenosine-receptor agonist, stimulated cAMP production in primary cultures of hepatocytes (Fig. 1, legend). As with glucagon stimulation, adenosine-receptor-stimulated cAMP production was increased after chronic ethanol exposure (Fig. 1b). When cells were treated with 0.1 μM-forskolin, a diterpene which stimulates adenylate cyclase independently of receptor occupancy [30], cAMP production was 71 % greater in cells cultured with 100 mM-ethanol for 48 h (288 ± 89 pmol/ 10^6 cells) than in controls (168 ± 48 ; $n = 4$, $P < 0.003$).

Since, unlike most other cell lines, primary cultures of hepatocytes metabolize ethanol [31,32], ethanol oxidation might be responsible for increased cAMP production. Activity of alcohol dehydrogenase was 1170 ± 250 units/ 10^6 cells in hepatocytes cultured for 48 h, compared with 1700 ± 200 ($n = 3$) in freshly isolated hepatocytes. If ethanol metabolism were responsible for ethanol-induced changes in receptor-dependent cAMP production, treatment of hepatocytes with 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase [33], should prevent any effects of ethanol on cAMP. However, pretreatment with 10 μM-4-methylpyrazole did not prevent the acute effects of ethanol on glucagon-receptor-dependent cAMP production (Fig. 3). Similarly, after co-incubation with 100 mM-ethanol and 10 μM-4-methylpyrazole for 48 h, glucagon-receptor-dependent cAMP levels were still increased to 238 ± 38 % ($n = 5$; $P < 0.05$) of those in cells not treated with ethanol. 4-Methylpyrazole alone had no effect on cAMP levels (results not shown). Treatment with 25 mM- and 50 mM-2-methylpropan-2-ol, a non-metabolizable alcohol [34], for 48 h also increased glucagon-receptor-dependent cAMP production to 125 ± 5 % and 163 ± 11 % of controls respectively ($n = 5$, $P < 0.05$).

In order to address more directly the effects of chronic ethanol treatment on adenylate cyclase, we measured cAMP production in membranes isolated from cells cultured without and with 100 mM-ethanol for 48 h. Basal activity of adenylate cyclase was equivalent between membranes isolated from control and ethanol-treated cells (Table 1). Treatment of membranes with 3 mM-MnCl₂, a direct stimulator of the catalytic subunit of adenylate cyclase [35], increased cAMP to the same extent in control and ethanol-treated cells (Table 1). However, stimulation

Table 1. Adenylate cyclase activity in membrane from control and ethanol-treated hepatocytes

Adenylate cyclase activity in membranes from control and ethanol-treated hepatocytes. Membranes were isolated from cells cultured in 0 or 100 mM-ethanol for 48 h and activity of adenylate cyclase measured as described in the Materials and methods section and expressed as pmol of cAMP/10 min per mg of protein ($n = 3-6$): * $P < 0.01$, † $P < 0.02$ compared with membranes from cells not cultured with ethanol.

	No ethanol	100 mM-ethanol, 48 h
Basal	53 ± 9	69 ± 14
3 mM-MnCl ₂	563 ± 48	540 ± 110
1 μM-Glucagon	101 ± 12	181 ± 12*
1 μM-Forskolin	306 ± 20	403 ± 50*
60 mM-NaF	321 ± 59	432 ± 48†

Fig. 4. Immunoblots of α_s and α_i proteins after chronic treatment with ethanol

Cells were cultured for 48 h with 0 or 100 mM-ethanol, membranes were isolated and immunoblots were prepared as described in the Materials and methods section. For α_i , lanes 1/2 and 3/4 contain 25 and 50 μg of membrane protein from control and ethanol-treated cells respectively. For α_s , lanes contain 100 μg of membrane protein. Immunoblots shown are representative of 8-9 experiments.

with 1 μM-glucagon and 1 μM-forskolin produced significantly higher levels of cAMP after chronic ethanol exposure (Table 1). Similarly, stimulation of cAMP production by 60 mM-NaF was greater in cells cultured with ethanol than in control cells. These results suggest that chronic ethanol treatment increased G-protein-mediated activation of adenylate cyclase, rather than increasing the activity of the catalytic subunit itself.

Adenylate cyclase activity is co-ordinately regulated by G_s and G_i . Therefore increased cAMP production could result from increased activation by G_s and/or decreased inhibition by G_i . The quantity of α_s and α_i proteins in membranes isolated from cells cultured without and with 100 mM-ethanol for 48 h was measured by Western blotting. By using antibody GA/1, which recognizes α_s proteins, no differences in α_s protein were detected between control hepatocytes and those chronically exposed to ethanol (Fig. 4). α_s protein quantity after chronic ethanol treatment was $114 \pm 9\%$ of control values ($n = 8$). However, immunoblot analysis using antibody AS/7, which recognizes the α_{i2} isoform present in hepatocytes [6], showed a significantly decreased quantity of α_{i2} protein in membranes from cells chronically treated with ethanol compared with control cells. After culture with 100 mM-ethanol for 48 h, the quantity of α_{i2}

Fig. 5. Loss of inhibitory effects of somatostatin and p[NH]ppG after chronic ethanol treatment

Membranes were isolated from cells cultured in 0 or 100 mM-ethanol for 48 h, and activity of adenylate cyclase was stimulated by 100 μM-forskolin (FSK) with and without 0.1 nM-p[NH]ppG or 1 μM-somatostatin (with 1 mg of bacitracin/ml). Forskolin-stimulated cAMP levels were 290 ± 31 pmol/mg of protein in control and 391 ± 70 in ethanol-treated ($n = 4$): * $P < 0.05$ compared with membranes not treated with inhibitors.

protein was decreased to $65 \pm 10\%$ ($P < 0.003$) of control values ($n = 9$).

We next determined whether decreased α_i protein was associated with decreased inhibitory function of G_i by measuring the ability of somatostatin and low concentrations of guanosine 5'-[βγ-imido]triphosphate (p[NH]ppG) [9] to inhibit forskolin-stimulated cAMP production in isolated membranes. In control membranes, 0.1 nM-p[NH]ppG inhibited forskolin-stimulated cAMP production by 23% (Fig. 5). However, in membranes from cells cultured in 100 mM-ethanol for 48 h, this effect was completely abolished (Fig. 5). Similarly, 1 μM-somatostatin decreased cAMP levels in control membranes by 47%, but had no effect in membranes from cells cultured with 100 mM-ethanol for 48 h (Fig. 5), suggesting that chronic exposure to ethanol decreased the function of G_i in hepatocytes.

DISCUSSION

Here we report that chronic exposure of primary cultures of rat hepatocytes to ethanol increases receptor-dependent cAMP production. This increase appears to result from decreased inhibition of adenylate cyclase by G_i . Loss of G_i function was associated with a decrease in the quantity of α_i protein in membrane fractions isolated from cells cultured with ethanol. Feeding of ethanol-containing diets to rats for 8 weeks has recently been reported to increase glucagon-receptor-dependent cAMP production in membranes isolated from liver homogenates [36]. These responses of hepatocytes to chronic ethanol treatment are distinct from the previously reported desensitization of receptor-dependent cAMP production in a variety of cultured cell lines (i.e. NG108-15 neuroblastoma × glioma [10,16,17] and S49 [18] cell lines), as well as in primary cultures of human lymphocytes [37] and rat cerebellum [38]. However, variations in cellular responses to ethanol have been previously reported [11,19]. For example, chronic exposure to ethanol has differential effects on α_s and α_i protein quantity in neural cell lines [11]. Such cell- and tissue-specific responses to chronic ethanol treatment may contribute to the diverse physiological effects of ethanol.

Acute and chronic exposure to ethanol alters hepatic metabolism, with the eventual appearance of alcoholic liver disease in 20% of all alcoholics [20,21]. The mechanism for these effects are not well understood. Because the liver is the primary site for ethanol metabolism, much of the research on the effects of

ethanol on liver function has focused on possible toxic effects of ethanol metabolites and the disruption of intermediary metabolism by products of ethanol metabolism [20,21]. Primary cultures of hepatocytes retain significant activity of alcohol dehydrogenase for several days in culture. However, the content of cytochrome *P*-450IIE1, an active catalyst for oxidation of ethanol, decreases rapidly in primary culture [39]. Therefore, primary cultures are a useful model system for distinguishing between direct effects of ethanol and toxic effects of ethanol metabolites produced via the alcohol dehydrogenase pathway. By inhibiting alcohol dehydrogenase with 4-methylpyrazole and utilizing the non-metabolizable tertiary alcohol 2-methylpropan-2-ol, we determined that both acute and chronic effects of ethanol on receptor-dependent cAMP production in hepatocytes were independent of ethanol metabolism via this pathway, suggesting that exposure to ethanol itself, rather than its metabolites, caused changes in cAMP production.

Chronic ethanol-induced increases in glucagon-receptor-dependent cAMP production in cultured hepatocytes were not due to an increased number of glucagon receptors on the cell surface (Fig. 2). Schuller *et al.* [40] also found that chronic ethanol feeding to rats did not change high-affinity binding of glucagon to hepatocytes. However, this is not the case for all receptors, since chronic ethanol feeding to rats decreases the number of epidermal-growth-factor and asialoglycoprotein receptors present on hepatocytes, possibly owing to impaired endocytosis after chronic exposure to ethanol [41].

Chronic exposure to ethanol had a heterologous effect on cAMP production, increasing the responsiveness of hepatocytes to more than one class of hormone. In addition to increased glucagon-receptor-stimulated cAMP production, adenosine-receptor-stimulated cAMP production was also increased after chronic exposure of hepatocytes to ethanol (Fig. 1*b*). Similarly, after chronic ethanol treatment, hepatocytes were more responsive to treatment with forskolin, a diterpene which stimulates cAMP production independently of receptor occupancy [30]. These results suggest that a common post-receptor component(s) in the cAMP production cascade is the molecular target for ethanol in hepatocytes.

cAMP production by adenylate cyclase activity is co-ordinately regulated by stimulation by G_s and inhibition by G_i . Chronic ethanol-induced increases in cAMP production could be due to changes in activity of adenylate cyclase itself or regulation by G-proteins. By utilizing isolated membrane fractions, activity of adenylate cyclase and regulation by G-proteins were directly examined. Chronic ethanol treatment did not appear to alter the activity of adenylate cyclase itself. Basal activity of adenylate cyclase was similar in membranes from control and ethanol-treated cells. Moreover, treatment of membrane fractions with Mn^{2+} , a direct activator of adenylate cyclase [35], increased cAMP levels to the same extent in membranes from control and ethanol-treated cells.

Chronic exposure to ethanol has been previously demonstrated to regulate the expression of α_s and α_i in various cultured neural cell lines. In NG108-15 neuroblastoma $\times$ glioma cells, heterologous desensitization of receptor-dependent cAMP production after chronic ethanol treatment is correlated with a decrease in α_s protein quantity, with no change in α_i [10,11]. In contrast, desensitization of N1E-115 neuroblastoma cells was correlated with an increase in α_i and no change in α_s [11]. After chronic exposure of primary cultures of hepatocytes to ethanol, increased receptor-stimulated cAMP production was associated with a $35 \pm 10\%$ decrease in α_i protein quantity, with no change in α_s protein (Fig. 4). These results suggest that ethanol causes a specific decrease in α_i protein quantity, rather than a generalized decrease in expression of G-proteins.

This decrease in α_i protein quantity was associated with decreased inhibitory activity in membranes from ethanol-treated cells. In control membranes, treatment with $1 \mu M$ -somatostatin decreased forskolin-stimulated cAMP production by 47%. After culture with 100 mM-ethanol for 48 h, this inhibitory effect of somatostatin was completely abolished (Fig. 5). This loss of somatostatin-receptor-dependent inhibition could be due to a decrease in receptor number. However, inhibition of forskolin-stimulated cAMP production by 0.1 nM -p[NH]ppG, which stimulates G_i activity [9], was also attenuated after chronic treatment with ethanol, suggesting that ethanol was acting at a post-receptor component of the inhibitory response. Taken together, these data suggest that chronic ethanol-induced increases in cAMP production reported here resulted from a loss of G_i function.

Decreased activity of G_i has been associated with increased production of cAMP under a number of conditions. For example, inhibition of G_i activity with pertussis-toxin treatment increases cAMP production in a number of cell types [4,42,43]. Similarly, treatment of S49 cells with the phorbol ester phorbol 12-myristate 13-acetate increases receptor-dependent cAMP production [4,43]. Bell & Brunton [4] demonstrated that this increase in cAMP production results from an inactivation of G_i function. In animal models of diabetes, decreased G_i function results in increased receptor-dependent stimulation of adenylate cyclase [6,9].

Chronic ethanol exposure was associated with a complete loss of somatostatin- and p[NH]ppG-mediated G_i function. This loss of function can only be partially accounted for by the 35% decrease in α_i protein. Therefore other mechanisms, such as regulation by covalent modification, must contribute to loss of G_i function. Activity of G_i can be decreased by protein kinase C-dependent phosphorylation [4,6]. In hepatocytes from diabetic animals, it has been suggested that loss of G_i function [9] results from decreased α_i -protein quantity [9], as well as protein kinase C-mediated phosphorylation of the α_i protein [6]. Since exposure of hepatocytes to ethanol transiently activates phospholipase C, mobilizes intracellular Ca^{2+} stores and activates protein kinase C [44], protein kinase C-dependent changes in G_i function might also contribute to increased receptor-stimulated cAMP production after chronic ethanol treatment. Similarly, rapid ethanol-induced phosphorylation of G_i might contribute to the acute stimulatory effects of 100–200 mM-ethanol on glucagon-receptor-stimulated cAMP production (Fig. 1*a*).

In summary, we have shown that chronic exposure of primary cultures of hepatocytes to ethanol decreases G_i -mediated inhibition of adenylate cyclase, resulting in increased receptor-stimulated cAMP production. This effect of ethanol on hepatocytes is distinct from the ethanol-induced heterologous desensitization of cAMP production observed in cultured neural cells and lymphocytes [10,16–18,37,38]. The mechanism for this cell-specific effect of ethanol on cAMP production in hepatocytes is not clear. α_{i2} , the predominant isoform of α_i in hepatocytes [6], is also found in NG108-15 neuroblastoma $\times$ glioma cells [45]. However, ethanol does not affect the expression of α_i in this neural cell line [10]. Cell-specific changes in G-protein quantity have been previously reported in other systems. For example, the decreased quantity of α_{i2} protein observed in animal models of diabetes is specific for parenchymal cells within the liver [6]. Cell-specific changes in G-protein quantity, such as the specific decrease in α_i protein quantity reported here, may be an important mechanism for the diversity of physiological responses to ethanol.

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