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Adenosine A_1 receptors mediate chronic ethanol-induced increases in receptor-stimulated cyclic AMP in cultured hepatocytes

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Cellular responses to adenosine depend on the distribution of the two adenosine receptor subclasses. In primary cultures of rat hepatocytes, adenosine receptors were coupled to adenylate cyclase via A_1 and A_2 receptors which inhibit and stimulate cyclic AMP production respectively. R-(−)-N^6-(2-phenylisopropyl)-adenosine (R-PIA), the adenosine A_1 receptor-selective agonist, inhibited glucagon-stimulated cyclic AMP production with an IC_{50} of 19 nM. This inhibition was blocked by the A_1-specific antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPDX). 5′-*N*-Ethylcarboxamidoadenosine (NECA), an agonist which stimulates A_2 receptors, increased cyclic AMP production with an EC_{50} of 0.6 μM. Treatment of primary cultures of rat hepatocytes with 100 mM ethanol for 48 h decreases the quantity and function of the inhibitory guanine-nucleotide regulatory protein (G_i), resulting in a sensitization of receptor-stimulated cyclic AMP production [Nagy and deSilva (1992) Biochem. J. **286**, 681–686]. When cells were cultured with 2 units/ml adenosine deaminase, to degrade extracellular adenosine, ethanol-induced increases in cyclic AMP production were completely prevented. Moreover, the specific A_1-receptor antagonist, CPDX, also blocked the chronic effects of ethanol on receptor-stimulated cyclic AMP production. Treatment with adenosine deaminase or CPDX also prevented the decrease in quantity of the α subunit protein of G_i observed in hepatocytes after chronic treatment with ethanol. Taken together, these results suggest that activation of adenosine A_1 receptors on primary cultures of hepatocytes is involved in the development of chronic ethanol-induced sensitization of receptor-stimulated cyclic AMP production.

INTRODUCTION

Exposure to ethanol disrupts hormone- and neurotransmitter-mediated signal transduction [1]. Evidence suggests that the chronic effects of ethanol on signal transduction are cell specific. For example, chronic exposure of neural cells and lymphocytes to ethanol decreases receptor-stimulated production of cyclic AMP (heterologous desensitization) [2–4]. This desensitization has been observed after ethanol exposure of cultured NG108-15 neuroblastoma × glioma and S49 lymphoma cells [2,4], as well as in rat cortex after long-term ethanol consumption [3] and peripheral lymphocytes from chronic alcoholics [5,6]. In contrast, increased receptor- and forskolin-stimulated cyclic AMP production has been reported in rat liver membranes after chronic alcohol consumption [7]. Similarly, chronic exposure of primary cultures of rat hepatocytes to ethanol increases receptor- and forskolin-stimulated cyclic AMP levels [8]. The mechanisms for these cell- and tissue-specific effects of ethanol are not well understood.

Adenosine is a locally acting hormone produced by a number of cells and tissues including hepatocytes, adipocytes and neural cells [9]. Adenosine interacts with several subclasses of plasma membrane receptors. Adenosine A_1 and A_2 receptors have been identified according to the order of potency for stimulation by different adenosine analogues [10]. Adenosine receptors are coupled to adenylate cyclase: A_1 receptors inhibit and A_2 receptors stimulate cyclic AMP production. In addition, in some cell types, A_1 receptors are coupled to ion channels, guanylate cyclase and phospholipase C [10]. Thus cellular responses to adenosine are dependent on the specific distribution of adenosine-receptor subtypes.

Continued exposure of cells to adenosine leads to a desensitization of receptor-mediated cyclic AMP production. In NG108-15 cells, which express stimulatory A_2 receptors, prolonged treatment with adenosine decreases receptor-stimulated cyclic AMP production [11]. In contrast, long-term treatment of adipocytes, which express inhibitory A_1 receptors, with adenosine decreases receptor-mediated inhibition of adenylate cyclase *in vivo* and in culture [12,13]. As a consequence of this desensitization of inhibitory responses, stimulation of cyclic AMP production by forskolin and isoprenaline is enhanced [12]. Using DDT_1 MF-2 cells, which express both A_1 and A_2 receptors, Ramkumar et al. [14] observed adenosine-induced desensitization of both receptor subtypes.

Adenosine is involved in many of the acute and chronic effects of ethanol in animals including motor inco-ordination, sedation and increased portal blood flow [15–17]. In cultured NG108-15 neuroblastoma × glioma cells, extracellular adenosine, interacting with adenosine A_2 receptors, mediates ethanol-induced decreases in stimulatory G-protein (G_S)-coupled cyclic AMP production [4]. In contrast, chronic ethanol-induced desensitization of receptor-stimulated cyclic AMP production in PC12 cells is independent of extracelluar adenosine concentrations [18]. Because of the cell-specific distribution of adenosine-receptor subtypes [10], it is possible that differences in distribution of adenosine A_1 and A_2 receptors contribute to the cell-specific effects of ethanol on receptor-dependent cyclic AMP production. We have investigated whether activation of adenosine A_1 receptors is required for the development of chronic ethanol-induced increases in cyclic AMP production in primary cultures of hepatocytes.

Abbreviations used: ADA, adenosine deaminase; CPDX, 8-cyclopentyl-1,3-dimethylxanthine; G_i, inhibitory guanine-nucleotide regulatory protein; G_S, stimulatory guanine-nucleotide regulatory protein; NECA, 5′-*N*-ethylcarboxamidoadenosine; R-PIA, R-(−)-N^6-(2-phenylisopropyl)adenosine; α_i, α subunit of G_i; α_S, α subunit of G_S.

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MATERIALS AND METHODS

Materials

RO20-1724 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. Antibodies to cyclic AMP, [^{3}H]cyclic AMP, ^{125}I-labelled cyclic AMP and ^{125}I-labelled anti-(rabbit IgG)antibodies were purchased from ICN. Antibodies to the α subunit of G_i (α_i) (AS/7) were purchased from New England Nuclear. Antibody to the α subunit of G_S (α_S) was purchased from Gramsch Laboratories, Germany. Adenosine deaminase (ADA) was purchased from Boehringer–Mannheim. R-(−)-*N*^{6}-(2-phenylisopropyl)-adenosine (R-PIA), 8-cyclopentyl-1,3-dimethylxanthine (CPDX), 5′-*N*-ethylcarboxamidoadenosine (NECA) and CGS-21680 were purchased from Research Biochemicals. All other reagent-grade chemicals were purchased from Sigma.

Hepatocyte isolation and culture

Hepatocytes were isolated by a modification of the method of Seglen as previously described [19,20]. Rats were anaesthetized with sodium pentobarbital and livers perfused via the portal vein 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% collagenase (type I) in Williams' E medium containing 10 mM Hepes at a flow rate of 15 ml/min. 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 6-well plates or T-75 culture flasks. After 2 h at 37 °C, non-adherent cells were removed by aspiration and the serum-containing medium replaced with Williams' E medium supplemented with 2 mM glutamine, 50 units/ml penicillin–streptomycin, 25 mM Hepes, 6.3 μg/ml insulin, 0.35 mM L-proline, 10 mM sodium pyruvate, 50 ng/ml epidermal growth factor, trace elements and 5 μg/ml linoleic acid complexed to fatty-acid-free BSA [18]. Gentamicin sulphate (0.5 μg/ml) was also included in the medium for some preparations. For chronic treatment with ethanol, 0 or 100 mM ethanol (final concn.) was added to the cell-culture medium after 24 h and cultured for a further 48 h. For cultures treated with 12.5 and 25 mM ethanol, ethanol was added to the medium after 2 h, medium was replaced after 48 h and assays conducted after 4 days in culture. 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 Carbopack C/0.2% Carbowax 1500 column. After 48 h in culture, 80 ± 5 mM ($n = 5$) of the 100 mM ethanol added to cell cultures remained in the medium. Cell number and viability was monitored by cell counting and Trypan Blue exclusion and did not differ between control and ethanol-treated cells (results not shown).

Concentration of adenosine in the culture medium after culture with and without 100 mM ethanol for 48 h was determined by preparing a fluorescent derivative of adenosine and quantification by h.p.l.c. [19]. Medium samples were pretreated by binding to phenylboronate to remove a contaminant in the cell-culture medium which interfered with h.p.l.c. analysis [4].

Receptor-dependent cyclic AMP production in intact cells

After culture in 0 or 100 mM ethanol for 48 h in 6-well plates, medium was removed by aspiration and cells washed once with 2 ml of phosphate-buffered saline containing 25 mM Hepes and 10 mM glucose (PBS). Cells were then pretreated for 10 min at 37 °C in 1 ml of PBS with 10 μM RO20-1724, an inhibitor of phosphodiesterase, and 0.4 or 2 units/ml ADA, to degrade any endogenously produced adenosine [21]. Various concentrations of agonist were then added and incubations continued for 10 min. Reactions were terminated with the addition of 100 μl of 1 M HCl and 2% (v/v) Nonidet P-40 and placed on ice for 30 min. Samples were stored at −20 °C and cyclic AMP determined by radioimmunoassay [21]. In some assays, adenosine concentrations in the incubation medium were measured by preparing a fluorescent derivative and quantified by h.p.l.c. [19]. Under the conditions for the cyclic AMP assays, adenosine was not detectable after treatment with 0.4 unit/ml ADA (results not shown).

Activity of cyclic AMP phosphodiesterase was measured in cells cultured with 0 or 100 mM ethanol for 48 h by the method of Thompson et al. [22]. Cells were homogenized in 25 mM Na_2PO_4, containing 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 17.5 μg/ml aprotinin and 4 mM 2-mercaptoethanol and the protein concentration adjusted to 5 mg/ml. Samples (200 μg of protein) were added to each assay tube containing 40 mM Tris/HCl, pH 8.0, with 4 mM 2-mercaptoethanol and 20 mM $MgCl_2$. Activity of phosphodiesterase was measured in the presence of 20 μM [^{3}H]cyclic AMP (0.04 mCi/mmol) over 10 min. Under these conditions, activity was linearly related to protein concentration from 100 to 800 μg of protein (results not shown).

Western-blot analysis of G proteins

Membranes were isolated [8] and resuspended at 1 mg of protein/ml. Samples (25 μg; for detection of α_i) and (75 μg; for detection of α_S) of membrane proteins were separated by SDS/PAGE using the Laemmli buffer system [23]. Proteins were then electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) non-fat dry milk in 10 mM Tris/HCl/0.9% NaCl/0.02% sodium azide, pH 7.0, (Tris/azide buffer) for 16 h, washed four times with 20 ml of Tris/azide buffer, and incubated with antisera [1:1000 dilution in 5% (w/v) non-fat dry milk] [8]. Membranes were again washed and probed with ^{125}I-labelled goat anti-(rabbit IgG) antibodies. Unbound ^{125}I-labelled goat anti-(rabbit IgG) was removed by washing, membranes dried and placed at −70 °C for autoradiography. Bands from control and ethanol-treated cells were compared by scanning densitometry. Densities of protein blots from treated samples were normalized to the density of the control sample run on the same blot. Under these conditions, absorptivity of labelled bands was linear from 10 to 100 μg of membrane protein for α_i and 25 to 150 μg of membrane protein for α_S (results not shown).

Data analysis

Values reported are means ± S.E.M. Data were analysed by paired *t*-test or the general linear model, using least-square means to test for differences between treatments. Data were logarithmically transformed as necessary before analysis to ensure a normal distribution. All statistical analyses were performed on the SAS statistical package for the personal computer. Best-fit curves from individual experiments were calculated using Table Curve (Jandel Scientific) and used to calculate IC_{50} and EC_{50} values. K_B values for antagonism of R-PIA inhibition of cyclic AMP production was calculated using the Schild equation [24].

RESULTS

A_1- and A_2-receptor agonists regulate cyclic AMP production in primary cultures of rat hepatocytes. NECA, an adenosine-receptor agonist active at both A_1 and A_2 receptors, stimulated cyclic AMP production with an EC_{50} of 0.6 ± 0.1 μM ($n = 3$) (Figure 1). After stimulation of cyclic AMP production with 50 nM glucagon, addition of the A_1-active agonist, R-PIA, inhibited cyclic AMP production with an IC_{50} of 18.7 ± 0.6 nM (Figure 2). Addition of 110 nM CPDX, a water-soluble A_1-selective antagonist, completely blocked the inhibitory effects of 10–100 nM R-PIA (Figure 2). The calculated K_B value for antagonism by CPDX was 6 nM, similar to reported values in the literature of 10.9 nM for A_1 receptors [25]. The A_2-selective agonist CGS-21680 did not inhibit cyclic AMP production at concentrations between 1 nM and 10 μM (results not shown). Inhibition of cyclic AMP production by A_1-receptor agonists was pertussis-toxin-sensitive. In control cells, 50 nM R-PIA inhibited cyclic AMP production by 48 ± 11 % ($n = 7$; $P < 0.05$). After treatment with 2 μg/ml pertussis toxin for 4 h, R-PIA did not significantly inhibit cyclic AMP production (18 ± 12 %, $n = 4$; $P = 0.31$).

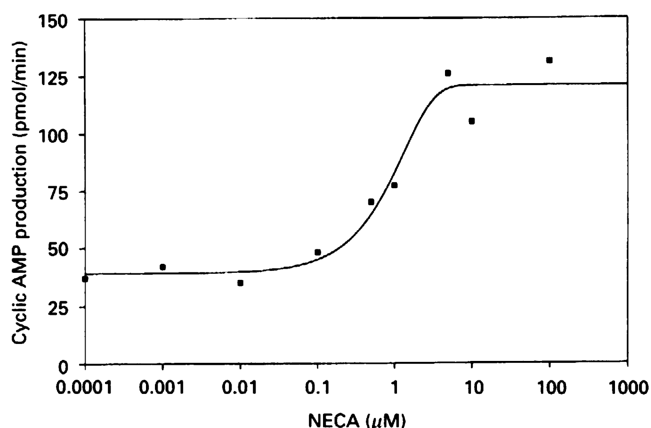

Figure 1 Stimulation of cyclic AMP production in primary cultures of hepatocytes with NECA, an A_2-receptor agonist

Cells were stimulated with 0–100 μM NECA for 10 min and cyclic AMP determined by r.i.a. as described in the Materials and methods section. The curve is representative of three experiments done in duplicate.

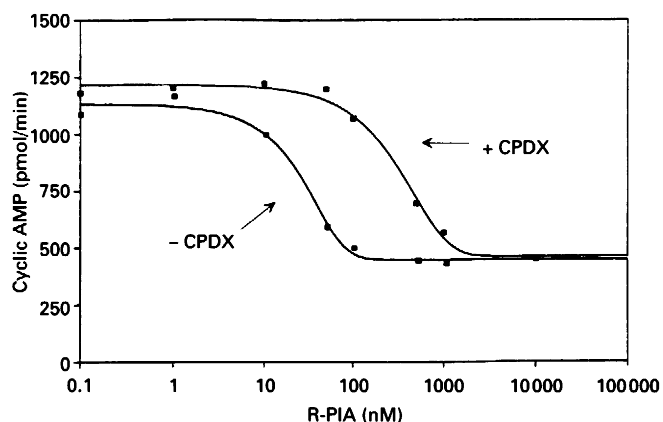

Figure 2 Effect of adenosine A_1 receptor agonist (R-PIA) and antagonist (CPDX) on glucagon-stimulated cyclic AMP production

Cells were pretreated for 5 min with 2 units/ml ADA in the presence and absence of 110 nM CPDX in PBS with 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Cells were then stimulated with 50 nM glucagon and 0–10 μM R-PIA for 10 min. Cyclic AMP was determined by r.i.a. Curves are representative of four experiments done in duplicate.

Treatment of primary cultures of rat hepatocytes with 50–200 mM ethanol for 48 h increases cyclic AMP production in response to stimulatory agonists [8]. Since many of the effects of ethanol are dependent on length of exposure as well as dose, we determined whether lower concentrations of ethanol over longer periods of exposure would also sensitize hepatocytes to stimulation by glucagon. After culture with 12.5 or 25 mM ethanol for 96 h, glucagon-stimulated cyclic AMP production was increased by 38–71 % with no change in basal cyclic AMP levels (Table 1). Increased production of cyclic AMP after ethanol treatment was not due to a decrease in total phosphodiesterase activity. Total hepatocyte PDE activity was 114 ± 25 pmol cyclic AMP hydrolysed/mg of protein per min in control cells and 107 ± 30 after culture in 100 mM ethanol for 48 h ($n = 7$; $P = 0.85$).

Adenosine can desensitize both stimulatory and inhibitory control of adenylate cyclase after prolonged exposure [11–14]. Primary cultures of hepatocytes release adenosine into their culture medium. Concentration of extracellular adenosine was 22.3 ± 4.4 nM in medium from control cells and 37.2 ± 11.7 nM in cells cultured in 100 mM ethanol for 48 h ($n = 3$; $*P < 0.05$ by paired t-test).

Since adenosine is present in the extracellular medium of hepatocyte cultures, we tested whether extracellular adenosine was required for ethanol-induced increases in receptor-stimulated cyclic AMP production. Cells were treated with 0.4 unit/ml ADA to metabolize extracellular adenosine to inosine, which is not a potent agonist for adenosine receptors [26]. Basal and receptor-stimulated cyclic AMP production was increased by 15–30 % after culture with ADA compared with control cells (Table 2). Cells treated with 100 mM ethanol for 48 h increased glucagon- and NECA-stimulated cyclic AMP production by 75 and 40 %, respectively, without increasing basal production of cyclic AMP. Co-culture of ethanol-treated cells with 0.4 unit/ml ADA prevented these ethanol-induced increases in glucagon- and NECA-stimulated cyclic AMP production (Table 2), suggesting that adenosine was involved in the chronic effects of ethanol on cyclic AMP in cultured hepatocytes.

Treatment with ADA prevents the interaction of adenosine with both A_1 and A_2 receptors present on the cell. In order to test whether adenosine was interacting specifically with A_1 receptors, hepatocytes were also cultured with 110 nM CPDX, a water-soluble adenosine-A_1-receptor antagonist (K_i 10.9 nM for A_1 receptors and 1440 nM for A_2 receptors) [25]. Culture of hepatocytes with CPDX had no effect on basal or receptor-stimulated cyclic AMP production (Table 2). Co-culture of ethanol-treated cells with 110 nM CPDX completely blocked ethanol-induced increases in receptor-stimulated cyclic AMP production (Table 2).

Since removal of extracellular adenosine, or blocking the interaction of adenosine with A_1 receptors with an antagonist, prevented the chronic effects of ethanol on cyclic AMP production, we next determined whether ethanol-induced decreases in $G\alpha_i$ could also be prevented by treatment with ADA or CPDX. Cells were co-cultured with 0.4 unit/ml ADA or 110 nM CPDX with and without 100 mM ethanol for 48 h. Quantities of $G\alpha_i$ and $G\alpha_s$ proteins in membranes was estimated by Western-

Table 1 Basal and glucagon-stimulated cyclic AMP production in cells cultured with 0–25 mM ethanol for 4 days

Hepatocytes were cultured with 0–25 mM ethanol for 4 days. Culture medium was removed and cells preincubated with 0.4 unit/ml ADA in PBS. Cells were then stimulated with and without 50 mM glucagon for 10 min. Values represent mean ± S.E.M., $n = 3$; $^{*}P < 0.05$ compared with cells not treated with ethanol.

	No ethanol	12.5 mM Ethanol	25 mM Ethanol
Basal	38 ± 4	41 ± 4	37 ± 5
50 nM Glucagon	136 ± 31	188 ± 31*	233 ± 46*

Table 2 Effects of ADA and CPDX on cyclic AMP production in cultured hepatocytes

n is shown in parentheses.

	Cyclic AMP production (pmol/mg of protein per min)		
	Basal	50 nM Glucagon	10 μM NECA
No ethanol	37 ± 4 (24)	255 ± 29 (24)	290 ± 55 (15)
Ethanol (100 mM)	42 ± 6 (24)	447 ± 51* (24)	403 ± 38† (15)
ADA (0.4 unit/ml)	82 ± 8‡ (7)	332 ± 51‡ (6)	335 ± 29‡ (6)
ADA (0.4 unit/ml) + ethanol (100 mM)	63 ± 8 (7)	344 ± 21 (5)	338 ± 47 (5)
CPDX (110 nM)	28 ± 3 (8)	29 ± 38 (9)	204 ± 36 (4)
CPDX (110 nM) + ethanol (100 mM)	26 ± 3 (8)	273 ± 48 (9)	229 ± 55 (4)

* $P < 0.001$, † $P < 0.01$ compared with no ethanol.
‡ $P < 0.05$ compared with no ethanol.

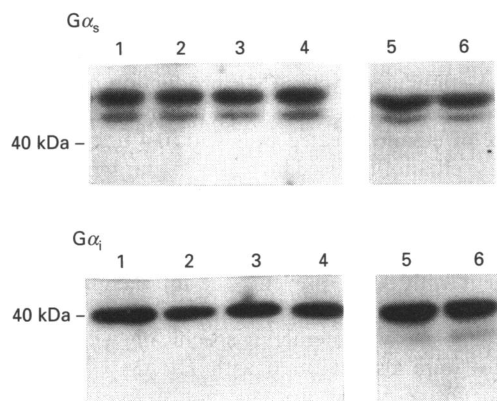

Figure 3 Effects of ADA and CPDX on Gα-protein quantity

Cells were cultured with (lanes 2, 4 and 6) and without (lanes 1, 3 and 5) 100 mM ethanol for 48 h with 0.4 unit/ml ADA (lanes 5 and 6) or 110 nM CPDX (lanes 3 and 4). Membranes were isolated and the quantity of $G\alpha_i$ and $G\alpha_S$ determined by Western blot. Figure is representative of experiments summarized in Table 3.

Table 3 Effects of ADA and CPDX on Gα-protein quantity

Values with different superscripts are significantly different ($P < 0.05$) by analysis of variance. *n* is shown in parentheses.

	Density (%)		
	No treatment	110 nM CPDX	0.4 unit/ml ADA
$G\alpha_S$			
No ethanol*	100[a] (15)	115 ± 15[a] (15)	93 ± 14[a] (6)
Ethanol (100 mM), 48 h†	99 ± 10[a] (15)	73 ± 6[a] (15)	73 ± 11[a] (6)
$G\alpha_i$			
No ethanol*	100[a] (15)	112 ± 27[ab] (15)	72 ± 14[b] (5)
Ethanol (100 mM), 48 h†	61 ± 8[b] (15)	89 ± 9[b] (15)	115 ± 20[ab] (5)

* Density expressed as a percentage of cells not treated with ADA or CPDX.
† Density expressed as a percentage of cells not treated with ethanol.

blot analysis (Figure 3). Treatment with 110 nM CPDX or ADA alone did not effect on $G\alpha_S$ protein quantity (Table 3). However, ADA significantly decreased the quantity of $G\alpha_i$ relative to control cells. This decrease in $G\alpha_i$ correlates with the increase in cyclic AMP production observed in hepatocytes treated with ADA (Table 2). Treatment with 100 mM ethanol for 48 h decreased the quantity of $G\alpha_i$ by 39%, but had no effect on $G\alpha_S$ (Table 3). Co-culture of ethanol-treated cells with either ADA or CPDX prevented this decrease in $G\alpha_i$ protein quantity (Table 3).

DISCUSSION

We have utilized a primary culture model for hepatocytes in order to investigate the mechanisms for the effects of ethanol exposure on cyclic AMP signal transduction. Treatment of primary hepatocyte cultures with ethanol over 2–4 days in culture results in increased responsiveness to receptor-stimulated cyclic AMP production (Table 1) [8]. A similar increase in glucagon- and forskolin-stimulated cyclic AMP production in liver membranes isolated from rats fed ethanol for 8 weeks has been reported [7]. As adenosine, interacting with adenosine A_2 receptors, has been found to mediate the decrease in cyclic AMP production observed in NG108-15 cells after chronic treatment with ethanol [4], we postulated that adenosine may also be involved in the effects of ethanol on cyclic AMP production in cultured hepatocytes. Here we report that the interaction of adenosine with A_1 receptors is required for the development of increased receptor-stimulated cyclic AMP production during culture with ethanol.

Previous investigations have reported that stimulation of adenosine A_2 receptors increases cyclic AMP production in isolated hepatocytes [27–29]. However, some investigators have been unable to detect A_2-receptor activity [30]. Adenosine, interacting with A_2 receptors, stimulates hepatic glycogenolysis [27,28,31] and increases system A amino acid transport [32]. Diaz et al. [33] also report that adenosine inhibits glucagon-stimulated cyclic AMP production in isolated hepatocytes. This inhibition required Ca^{2+} in the incubation medium [33]. In primary culture of hepatocytes, activity of both A_1 and A_2 receptors was detected. Because cultured hepatocytes release adenosine into their incubation medium [19], activity of adenosine receptors was measured after pretreatment of hepatocytes with ADA to remove endogenously produced adenosine. Under these conditions, NECA, an agonist which activates both A_1 and A_2 receptors, increased cyclic AMP production, while R-PIA, an A_1-receptor agonist, decreased glucagon-stimulated cyclic AMP production. The A_1-selective antagonist, CPDX, reduced the inhibitory effects

of R-PIA. The ability of pertussis toxin to block inhibition of cyclic AMP production by R-PIA provides further support for the presence of A_1 receptors coupled to G_i in these cells. Taken together, these functional assays demonstrate that adenosine receptors are coupled to both stimulation and inhibition of cyclic AMP production in primary cultures of rat hepatocytes.

Prolonged exposure of cells to adenosine results in a desensitization of receptor-mediated stimulation and/or inhibition of cyclic AMP production [11–14,34]. Desensitization resulting from activation of A_2 receptors decreases receptor-stimulated cyclic AMP production [34,35]. In contrast, activation of A_1 receptors results in a desensitization to inhibitory signals and, consequently, increases cyclic AMP production in response to stimulatory hormones [12]. During hepatocyte culture, 20–25 nM adenosine was present in the medium of control cells. Removal of this adenosine by treatment with ADA increased basal and receptor-stimulated cyclic AMP production, and also decreased $G\alpha_i$ protein quantity. In contrast, culture with CPDX, to prevent interaction with A_1 receptors, did not change cyclic AMP levels in control cells. Green [36] reports similar increases in cyclic AMP production after treatment of cultured neuroblastoma cells with ADA and suggests that the concentration of endogenously released adenosine (12 nM) was sufficient to desensitize cyclic AMP production during culture [36]. These responses suggest that the adenosine in the medium of hepatocyte cultures acts to regulate tonically the capacity of the hepatocyte for cyclic AMP production. Extracellular adenosine was also required for ethanol-induced increases in receptor-stimulated cyclic AMP production. However, both ADA and CPDX prevented ethanol-induced responses, indicating that these increases in cyclic AMP production were mediated via the specific interaction of adenosine with the A_1 receptor.

While the data reported here indicate that activation of adenosine A_1 receptors is necessary for the development of ethanol-induced increases in cyclic AMP production, they do not suggest that activation of A_1 receptors is sufficient for these responses. Ethanol treatment increased the concentration of adenosine in the medium of hepatocyte cultures. However, this small difference cannot account for the changes in cyclic AMP production reported here. Moreover, in cultured hepatocytes, changes in cyclic AMP production in response to adenosine alone, as demonstrated by the increase in cyclic AMP production after treatment of control cultures with ADA, were distinct from responses to adenosine and ethanol in combination. In other cell types, ethanol facilitates the stimulation of adenylate cyclase by G_S [37,38]. It is possible that ethanol modifies the effects of adenosine on the A_1-receptor-mediated inhibition of cyclic AMP production. Recent evidence from our laboratory indicates that ethanol can enhance the inhibitory effects of R-PIA on cyclic AMP levels in hepatocytes [38a]. An increase in the cellular responsiveness to adenosine via the A_1 receptor in the presence of ethanol could contribute to the long-term increases in cyclic AMP production reported here.

The molecular mechanisms for heterologous desensitization/sensitization of cyclic AMP production vary between cell types. Desensitization of receptor-stimulated cyclic AMP production after chronic ethanol treatment in NG108-15 cells is correlated with decreased activity and quantity of $G\alpha_S$ [2]. Prolonged activation of adenosine A_2 receptors in PC12 cells is also correlated with decreased $G\alpha_S$ protein, as well as an increase in phosphodiesterase activity [39]. In contrast, increased cyclic AMP production observed after activation of A_1 receptors in adipocytes results from a decrease in $G\alpha_{i1,2}$ protein quantity [34]. Increased receptor-stimulated cyclic AMP production in primary cultures of hepatocytes after chronic ethanol treatment was associated with a decrease in $G\alpha_i$ protein quantity (Table 3) [8]. However, additional mechanisms, such as changes in the functional activity of G proteins or activity of adenylate cyclases, may also contribute to increased responsiveness to stimulatory hormones. In chick hepatocytes, desensitization of cyclic AMP production is associated with a decrease in the ability of G_S to activate adenylate cyclase [40]. Direct activation of adenylate cyclase with $MnCl_2$ in isolated membranes does not differ between control and ethanol-treated hepatocytes [8], indicating that adenylate cyclase is not a direct target for ethanol action. However, altered G protein function after ethanol treatment could contribute to changes in the activation of adenylate cyclase isoforms in the hepatocyte.

Increased activity of phosphodiesterase is involved in desensitization in PC12 cells after activation of adenosine A_{2a} receptors for 4–24 h [39]. In contrast, prolonged inhibition of cyclic AMP production in NG108-15 cells decreases the rate of cyclic AMP degradation, resulting in increased cyclic AMP levels in response to stimulatory agonists [41]. Liver expresses several isoenzymes of phosphodiesterase [42,43]. Measurements of cyclic AMP production in this study were conducted in the presence of RO20-1724, a specific inhibitor of phosphodiesterase IV. Therefore, decreased activity of other phosphodiesterase isoforms in the hepatocyte could have contributed to the increased cyclic AMP observed after chronic ethanol treatment. However, total activity of phosphodiesterases in primary cultures of hepatocytes was not affected by culture with ethanol, suggesting that sensitization of cyclic AMP production did not result from decreased phosphodiesterase activity.

In summary, chronic treatment of primary cultures of hepatocytes with ethanol results in an increase in receptor-stimulated cyclic AMP production. This increase is associated with a decrease in quantity of $G\alpha_i$ protein and requires the interaction of adenosine with adenosine A_1 receptors. Together with previous investigations in NG108–15 cells, which demonstrate that ethanol-induced heterologous desensitization of receptors coupled to G_S is mediated via adenosine A_2 receptors [4], these results suggest that the differential distribution of adenosine receptor subtypes may contribute to cell- and tissue-specific effects of ethanol on cyclic AMP signal transduction.

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