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# ROLE OF ADENOSINE $A_1$ RECEPTORS IN INHIBITION OF RECEPTOR-STIMULATED CYCLIC AMP PRODUCTION BY ETHANOL IN HEPATOCYTES

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**Abstract**—Brief exposure of primary cultures of hepatocytes to ethanol had a biphasic effect on glucagon receptor-dependent cyclic AMP (cAMP) production: 25–50 mM ethanol decreased cAMP levels, whereas treatment with 100–200 mM ethanol increased cAMP. This biphasic effect was also observed after pretreatment with 10 $\mu$M 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Adenosine $A_1$ and $A_2$ receptors in primary cultures of rat hepatocytes are coupled to inhibition and stimulation of adenylyl cyclase, respectively. Since primary cultures of hepatocytes release adenosine into their extracellular media, we tested whether the acute effects of ethanol on cAMP were mediated by extracellular adenosine. Co-incubation with 2 U/mL adenosine deaminase prevented inhibition of cAMP production by 25–50 mM ethanol, but had no effect on stimulation by 100–200 mM ethanol. Pretreatment of hepatocytes with 110 nM 8-cyclopentyl-1,3-dimethylxanthine, an adenosine $A_1$ receptor antagonist, also completely blocked the inhibitory effects of ethanol on cAMP production. Low concentrations of ethanol enhanced the inhibitory effects of $R(-)N^6$-(2-phenylisopropyl)adenosine, an $A_1$ receptor agonist, on cAMP production in cells pretreated with adenosine deaminase to remove endogenous adenosine. These data suggest that endogenously produced adenosine can be an important modulator of the effects of ethanol on receptor-stimulated cAMP production in primary cultures of rat hepatocytes.

*Key words:* ethanol; cAMP; hepatocytes; adenosine; guanine nucleotide regulatory proteins; adenosine receptors

Acute and chronic exposures to ethanol have profound effects on cAMP† production in a number of cultured cells, as well as in the whole animal [1]. In membranes isolated from a variety of cell types, including S49 lymphoma cells [2] and striatal [3] and cerebral cortical tissues [4], ethanol increases receptor-stimulated cAMP production. Ethanol appears to enhance receptor-stimulated cAMP production by increasing the coupling of activated $G_S$ to adenylyl cyclase [5, 6].

In contrast to this acute stimulation of cAMP production consistently observed in membrane preparations, responses of intact cells to ethanol are more variable. In NG108-15 neuroblastoma × glioma cells [7], N1E-115 neuroblastoma cells [8], as well as isolated human lymphocytes [9], ethanol increases receptor-stimulated cAMP production. However, ethanol has also been reported to inhibit forskolin-stimulated cAMP production in N1E-115 cells [10]. Using two subclones of the rat pheochromocytoma cell line (PC 12), Rabe *et al.* [11] observed that ethanol inhibited receptor-dependent cAMP production in one subclone, yet stimulated cAMP levels in another. These studies suggest that the effects of ethanol on the intact cell may involve mechanisms in addition to the direct effects of ethanol on $G_S$ previously reported in isolated membranes [2–4]. Interactions between a variety of cross-regulatory pathways may contribute to the final observed changes in cAMP production.

The locally acting hormone, adenosine, may contribute to the effects of ethanol on cellular cAMP production in intact cells. Recent evidence suggests that adenosine is an important mediator for many of the acute and chronic effects of ethanol. Several cell types have been shown to release adenosine into the extracellular space [12–14]. After a brief treatment with ethanol, increases in extracellular adenosine have been observed in neural cells, lymphocytes [12], PC 12 cells [13] and hepatocytes [14]. Moreover, intubation of rats with ethanol increased plasma concentrations of adenosine [15]. Extracellular adenosine, interacting with adenosine receptors on the cell surface, is thought to mediate ethanol-induced sedation [16], motor incoordination [17], and increases in portal blood flow [15]. In NG 108-15 [12] and PC 12 [13] cells, ethanol-induced increases in extracellular adenosine result in a stimulation of cAMP production. Adenosine also mediates the chronic effects of ethanol on receptor-stimulated cAMP production in some cell types, including NG108-15 cells [12] and hepatocytes [18]. In contrast, chronic effects of ethanol on receptor-

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† Abbreviations: ADA, adenosine deaminase; cAMP, cyclic AMP; CPDX, 8-cyclopentyl-1,3-dimethylxanthine; $G_i$, inhibitory guanine nucleotide regulatory protein; $G_s$, stimulatory guanine nucleotide regulatory protein; NECA, 5′-*N*-ethylcarboxamidoadenosine; PC 12, rat pheochromocytoma cells; and PIA, $R(-)N^6$-(2-phenylisopropyl)-adenosine.

mediated cAMP production appear to be independent of extracellular adenosine in PC 12 cells [13].

Cellular responses to adenosine are dependent on the distribution of adenosine receptor subtypes. Adenosine receptors are coupled to adenylyl cyclase, with $A_1$ receptors inhibiting cAMP production and $A_2$ receptors stimulating cAMP [19]. $A_1$ receptors can also be coupled to guanylyl cyclase, ion channels and phospholipase C [19]. Endogenously produced extracellular adenosine, acting via inhibitory $A_1$ or stimulatory $A_2$ receptors, might therefore contribute to the differential effects of ethanol on cAMP production in intact cells. We have observed that primary cultures of hepatocytes express activity of both $A_1$ and $A_2$ receptors [18]. In the present study, the role of adenosine in mediating the acute effects of ethanol on cAMP production in primary cultures of hepatocytes was investigated.

## MATERIALS AND METHODS

*Materials.* RO 20-1724 was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Cell culture reagents were purchased from the Grand Island Biological Co. (Burlington, Ontario) and ICN (Dorval, Quebec). Antibody to cAMP and [$^{125}$I]cAMP were purchased from ICN (Dorval, Quebec). Adenosine deaminase was purchased from Boehringer Mannheim (Dorval, Quebec). Adenosine receptor agonists and antagonists were purchased from Research Biochemicals, Inc. (Natick, MA). All other reagent grade chemicals were purchased from Sigma (St. Louis, MO).

*Hepatocyte isolation and culture.* Male Wistar rats (200–300 g) were allowed free access to Purina rat chow and water. Hepatocytes were isolated by a modification of the method of Seglen [20], as previously described [21]. Rats were anaesthetized with sodium pentobarbital, and livers were 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 with 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 containing 7.5% fetal bovine serum. Cells were plated at $2 \times 10^5$ cells/mL in collagen-coated 6-well plates. After 2 hr at 37°, non-attached cells were removed by aspiration and the serum containing medium was replaced with Williams E medium supplemented with 2 mM glutamine, 50 U/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, selenium and 5 μg/mL linoleic acid complexed to fatty-acid free bovine serum albumin [21]. Gentamycin sulfate (0.5 μg/mL) was also included in the medium for some preparations. Cell number and viability were monitored by cell counting and trypan blue exclusion.

*Receptor-dependent cAMP production in intact cells.* After culture for 72 hr, medium was removed by aspiration and cells were washed once with 2 mL of PBS with 25 mM HEPES and 10 mM glucose. Cells were then pretreated for 10 min at 37° with 10 μM RO 20-1724, an inhibitor of phosphodiesterase, and 2 U/mL ADA, to degrade any endogenously produced adenosine [7] or 10 μM 4-methylpyrazole, to inhibit alcohol dehydrogenase. The efficacy of ADA treatments in removing extracellular adenosine was monitored by measuring the concentration of adenosine in incubation media from cells treated with and without ADA. A fluorescent derivative of adenosine was prepared and quantified by HPLC [14]. Various concentrations of agonist were then added and incubations continued for 10 min. Reactions were terminated with the addition of 100 μL of 1 N HCl and 2% Nonidet P-40 and placed on ice for 30 min. Samples were stored at −20° and cAMP was determined by radioimmunoassay [7].

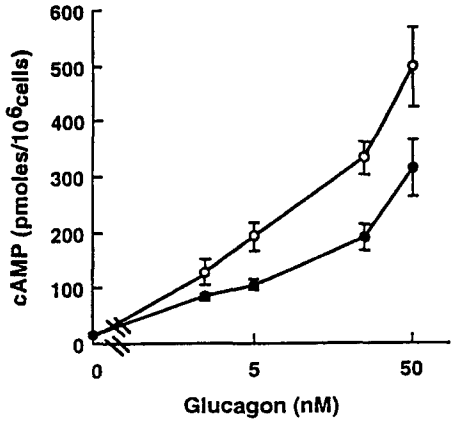


Fig. 1. Acute effects of ethanol on glucagon receptor-stimulated cAMP production. Primary cultures of rat hepatocytes were stimulated with 0–50 nM glucagon in the presence (●) and absence (○) of 25 mM ethanol, and cAMP levels were determined as described in Materials and Methods. Values are means ± SEM, N = 3. Glucagon increased cAMP over basal levels ($P < 0.001$) and ethanol decreased cAMP compared with cells not treated with ethanol ($P < 0.001$).

*Statistical analysis.* Values reported are means ± SEM. Data were analyzed by the general linear models procedure using Dunnett's test to measure differences between treatments on SAS for personal computers.

## RESULTS

Stimulation of primary cultures of rat hepatocytes with glucagon resulted in a concentration-dependent increase in cAMP production (Fig. 1). Treatment with 25 mM ethanol had no effect on basal levels of cAMP, but significantly decreased cAMP levels at all concentrations of glucagon tested (Fig. 1). Increasing ethanol concentrations had a biphasic effect on cAMP production in the presence of a submaximally active concentration of glucagon. Concentrations of 25–50 mM ethanol inhibited glucagon-receptor-dependent cAMP production, whereas 100–200 mM ethanol, stimulated cAMP production (Fig. 2). When cells were preincubated with 10 μM 4-methylpyrazole, an inhibitor of alcohol

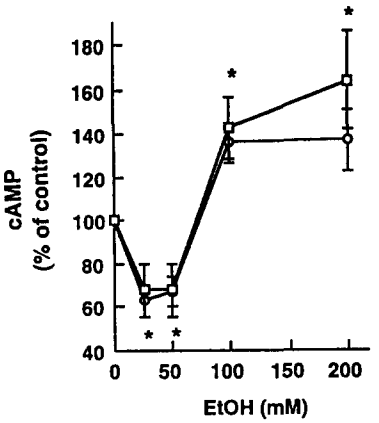


Fig. 2. Biphasic effect of ethanol on glucagon receptor-stimulated cAMP production. Primary cultures of rat hepatocytes were preincubated in the presence (□) and absence (○) of 10 $\mu$M 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Cells were then stimulated with 50 nM glucagon and 0–200 mM ethanol. cAMP levels were determined as described in Materials and Methods. cAMP levels in the absence of ethanol were 432 ± 79 and 299 ± 70 pmol/$10^6$ cells in the absence and presence of 4-methylpyrazole, respectively. Values are expressed as a percentage of cAMP in control cells that were not treated with ethanol and represent means ± SEM, N = 3–5. Key: (*) $P < 0.05$, compared with cells not treated with ethanol.

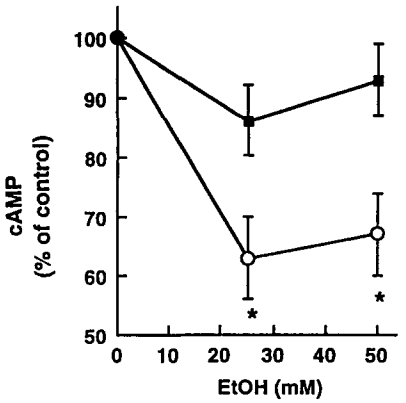


Fig. 4. Effect of an adenosine $A_1$ receptor antagonist, CDPX, on ethanol-induced inhibition of cAMP. Cells were preincubated with (■) and without (○) 110 nM CPDX, and then were stimulated with 50 nM glucagon and 0–50 mM ethanol. Glucagon-stimulated cAMP production in the absence of ethanol was 162 ± 38 and 148 ± 45 pmol/$10^6$ cells in the absence and presence of CPDX, respectively. Values are expressed as a percentage of cAMP in control cells that were not treated with ethanol and represent means ± SEM, N = 3. Key: (*) $P < 0.05$, compared with cells not treated with ethanol.

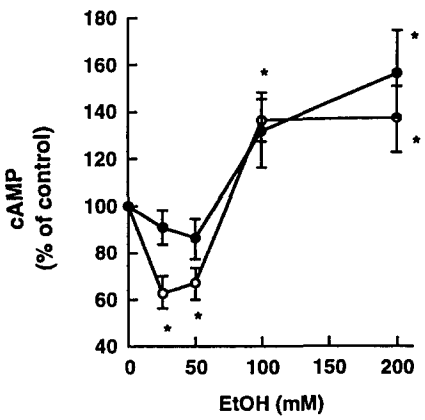


Fig. 3. Effect of adenosine deaminase (ADA) on ethanol-induced changes in cAMP. Cells were preincubated in the presence (●) or absence (○) of 2 U/mL ADA. Then the cells were stimulated with 50 nM glucagon and 0–200 mM ethanol, and cAMP production was determined. cAMP production in the absence of ethanol was 138 ± 29 and 112 ± 19 pmol/$10^6$ cells with and without ADA, respectively. Values are expressed as a percentage of cAMP in control cells that were not treated with ethanol and represent means ± SEM, N = 6. Key: (*) $P < 0.05$, compared with cells not treated with ethanol.

dehydrogenase, this biphasic effect of ethanol on glucagon-stimulated cAMP production was still observed (Fig. 2), suggesting that the effects of ethanol on cAMP production were independent of ethanol oxidation via alcohol dehydrogenase.

Primary cultures of hepatocytes release adenosine into the extracellular media [14]. Treatment of primary cultures of rat hepatocytes with 12.5 to 200 mM ethanol for 6 min increases extracellular adenosine concentrations [14]. If this endogenously produced adenosine were interacting with adenosine receptors on the hepatocytes, cAMP production might be altered as a consequence. Cellular responses would be dependent on the type of adenosine receptor present on the membrane, as well as the local concentrations of adenosine produced. To determine if extracellular adenosine was modulating glucagon-receptor-stimulated cAMP production, cells were co-incubated with 2 U/mL ADA and increasing concentrations of ethanol. ADA metabolizes adenosine to inosine, which does not interact with adenosine receptors. Adenosine concentrations were 0.14 ± 0.03 $\mu$M (N = 3) in cells not treated with ADA. After treatment with 2 U/mL ADA, adenosine was not detectable in the incubation medium. Figure 3 illustrates that removal of extracellular adenosine prevented the inhibitory effects of low concentrations of ethanol, but had no effect on the stimulatory effects at higher concentrations. However, when cells were stimulated with NECA, an agonist active at $A_2$ receptors, higher concentrations of ethanol were able to increase cAMP production. cAMP production in cells treated with 10 $\mu$M NECA was 336 ± 47 pmol/$10^6$ cells (N = 8). Addition of 200 mM ethanol increased NECA-stimulated cAMP production to 160 ± 20% of control ($P < 0.05$).

If adenosine interaction with $A_1$ receptors is required for inhibition of cAMP production at low concentrations of ethanol, then treatment with CPDX, an $A_1$ specific antagonist ($K_i$ = 11 nM), should prevent the decrease in cAMP levels. Cells

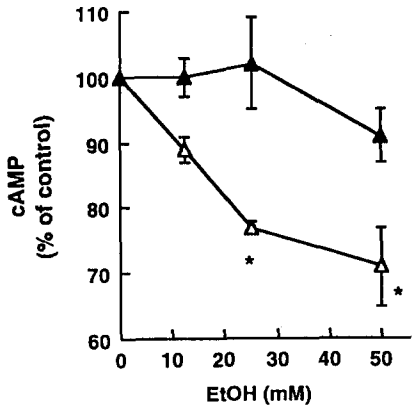


Fig. 5. Effect of CPDX on ethanol-induced inhibition of forskolin-stimulated cAMP production. Cells were preincubated with (▲) and without (△) 110 nM CPDX and then were stimulated with 0.1 μM forskolin and 0–50 mM ethanol. Forskolin-stimulated cAMP levels in the absence of ethanol were 433 ± 83 and 367 ± 65 pmol/$10^6$ cells in the presence and absence of CPDX, respectively. Values are expressed as a percentage of cAMP in control cells that were not treated with ethanol and represent means ± SEM, N = 3–4. Key: (*) $P < 0.05$, compared with cells not treated with ethanol.

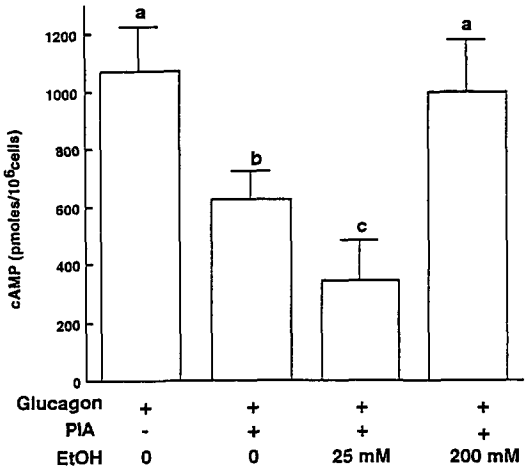


Fig. 6. Effect of ethanol on inhibition of cAMP production by PIA. Cells were pretreated with 2 U/mL ADA in PBS containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$. cAMP production was stimulated with 50 mM glucagon in the presence and absence of 10 nM PIA and ethanol. Values represent means ± SEM, N = 4. Values with different superscripts are significantly different, $P < 0.05$.

were co-incubated with 110 nM CPDX and 0–50 mM ethanol. CPDX completely prevented ethanol-induced inhibition of glucagon-stimulated cAMP production (Fig. 4). Low concentrations of ethanol had similar inhibitory effects on forskolin-stimulated cAMP production. Forskolin is a diterpene that stimulates cAMP production via interactions with adenylyl cyclase and $G_S$. Concentrations of ethanol as low as 12.5 mM inhibited forskolin-stimulated cAMP production (Fig. 5). Pretreatment with 110 nM CPDX completely blocked inhibition of cAMP production by ethanol (Fig. 5).

These results suggest that the presence of adenosine in the extracellular media is required for inhibition of receptor-stimulated cAMP production by ethanol. However, they do not distinguish whether ethanol-induced increases in extracellular adenosine are required for this response and/or whether ethanol acts by enhancing the ability of adenosine to inhibit adenylyl cyclase. To address this question, cells were pretreated with 2 U/mL ADA to remove endogenous adenosine and cAMP production was stimulated with glucagon. Treatment with 10 nM PIA, an adenosine $A_1$ receptor agonist that is not metabolized by ADA, reduced cAMP production (Fig. 6). The addition of 25 mM ethanol further decreased cAMP levels. This reduction was observed even in the presence of ADA, which prevents accumulation of endogenously produced adenosine, suggesting that low concentrations of ethanol enhanced the inhibitory effects of adenosine $A_1$ receptor agonists. In contrast, 200 mM ethanol completely prevented inhibition of cAMP production by PIA (Fig. 6).

## DISCUSSION

Gorman and Bitensky [22] first demonstrated that ethanol stimulates receptor-stimulated cAMP production in membranes isolated from hepatocytes. Ethanol has since been shown to increase receptor-stimulated cAMP production in membranes isolated from a variety of tissues and cells by enhancing the interaction between hormone-receptor and G-protein and the subsequent activation of adenylyl cyclase [5, 6]. However, investigations with intact cells indicate that additional regulatory factors contribute to the acute actions of ethanol on cAMP production. Rabe *et al.* [11], using two subclones of PC12 cells, found that the effects of ethanol on the intact cell are distinct from those in isolated membranes. In whole cells, ethanol inhibits or stimulates receptor-dependent cAMP production in different subclones, whereas cAMP production in membranes is increased by ethanol in both subclones [11]. Here we report that ethanol has a biphasic effect on receptor-stimulated cAMP production in intact hepatocytes, with low concentrations of ethanol inhibiting cAMP production and higher concentrations increasing cAMP. Primary cultures of hepatocytes can metabolize ethanol via the alcohol dehydrogenase pathway [21]. However, the biphasic effect of ethanol on cAMP was observed even when cells were pretreated with 4-methylpyrazole, an inhibitor of alcohol dehydrogenase, indicating that this response was not dependent on ethanol metabolism.

Acute exposure to ethanol inhibits adenosine uptake via the nucleoside transporter and, as a consequence, increases extracellular adenosine concentrations in a variety of cell types [12–14]. Adenosine is a locally acting hormone that can modulate the activity of adenylyl cyclase: adenosine $A_1$ receptors inhibit cAMP production, whereas $A_2$ receptors stimulate cAMP production. Previous

investigators have demonstrated the presence of adenosine $A_2$ receptors on hepatocytes [23]. Adenosine, interacting with these receptors, stimulates hepatic glycogenolysis [24–26] and increases system A amino acid transport [27]. Diaz *et al.* [28] also reported that adenosine inhibits glucagon-stimulated cAMP production in isolated hepatocytes. We have observed that adenosine receptor agonists stimulate and inhibit cAMP production in primary cultures of hepatocytes, indicating that both $A_1$ and $A_2$ receptors are active in this system [18].

Extracellular adenosine, interacting with adenosine $A_1$ and/or $A_2$ receptors, could contribute to the biphasic effects of ethanol on receptor-stimulated cAMP production in hepatocytes. Co-incubation of hepatocytes with 2 U/mL ADA during ethanol treatment completely prevented the inhibitory effects of ethanol, suggesting that interaction of adenosine with $A_1$ receptors was modulating glucagon-stimulated cAMP production. CPDX, an adenosine $A_1$ receptor antagonist, also completely blocked the inhibitory effects of ethanol on glucagon- and forskolin-stimulated cAMP production (Figs. 4 and 5). These results suggest that adenosine is required for ethanol inhibition of cAMP production in primary cultures of hepatocytes.

Endogenously produced adenosine does not appear to be involved in stimulation of cAMP production at higher concentrations of ethanol. This lack of response contrasts to the role of adenosine in stimulating cAMP production after acute ethanol treatment in NG108-15 [12] and PC12 [13] cells. When hepatocytes were stimulated with 10 $\mu$M NECA, 200 mM ethanol increased cAMP production, indicating that ethanol can enhance $A_2$ receptor-mediated responses. Maximal stimulation of cAMP production by glucagon in primary cultures of hepatocytes occurs at approximately 1 $\mu$M (Nagy LE and deSilva SEF, unpublished observations). In these studies, cells were stimulated with a submaximal concentration of glucagon in order to assess the ability of ethanol to both increase and decrease cAMP production. Therefore, if endogenously produced adenosine were stimulating $A_2$ receptors after ethanol treatment, the cells should still have the capacity to increase cAMP production. However, removal of adenosine with ADA had no effect on the stimulation of cAMP production observed at 100–200 mM ethanol. The $EC_{50}$ for stimulation of cAMP production by NECA, an adenosine receptor agonist, is 0.6 $\mu$M in primary cultures of hepatocytes [18]. These results indicate that, while ethanol can enhance adenosine $A_2$ receptor-stimulated cAMP production, the quantity of endogenously produced adenosine under the conditions used in this assay (0.14 $\mu$M) was insufficient to activate adenosine $A_2$ receptors.

Increased cAMP production at 100–200 mM ethanol may be due to an enhanced interaction between $G_S$ and adenylyl cyclase [5, 6]. It is possible that increased coupling of $G_S$ to adenylyl cyclase by ethanol [5, 6] is not detectable at concentrations of ethanol less than 100 mM in these cells. Indeed, Gorman and Bitensky [22] did not see enhancement of glucagon-stimulated cAMP production at less than 300 mM ethanol. Similarly, Whetton *et al.* [29] only observed increases in glucagon-stimulated cAMP production in hepatocyte membranes at concentrations of ethanol greater than 100 mM. Alternatively, at higher concentrations of ethanol, increased cAMP production in intact hepatocytes may be due to a loss of $G_i$ function, as well as a stimulation of $G_S$ activation of adenylyl cyclase. In primary cultures of rat hepatocytes, 200 mM ethanol completely blocked the ability of PIA to inhibit cAMP production (Fig. 6). This loss of inhibition could be due to an increase in the activation of $G_S$ and/or a decrease in inhibition by $G_i$. In membranes isolated from rat brain cortex, *in vitro* treatment with 50–200 mM ethanol reduces the ability of PIA to decrease cAMP production [30]. Bauché *et al.* [30] suggest that this loss of inhibition is due to a direct or indirect impairment of $G_i$-mediated inhibition of adenylyl cyclase in cortical tissue [30]. However, in striatal membranes, ethanol has no effect on inhibitory control of adenylyl cyclase [31, 32]. These reported differences in the effect of ethanol on inhibition of adenylyl cyclase may be due to differences in the regulatory properties of the multiple isoforms of adenylyl cyclase [33].

In contrast to the loss of PIA inhibition of cAMP production at 200 mM ethanol, lower concentrations of ethanol enhanced the ability of PIA to inhibit cAMP function. This increase in inhibitory response to PIA occurred independently of any changes in extracellular adenosine concentration. While the mechanism for this enhanced inhibition of adenylyl cyclase is unknown, it is possible that increased activity of $G_i$ after treatment with low concentrations of ethanol may function analogously to the facilitation of $G_S$ interaction with adenylyl cyclase observed at higher concentrations of ethanol [5, 6]. Thus, these results suggest that inhibition of receptor-stimulated cAMP production by ethanol in primary cultures of hepatocytes results from the interaction of extracellular adenosine with $A_1$ receptors, as well as an enhancement of the ability of adenosine receptor agonists to decrease cAMP production.

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