0026-895X/91/050812-06$03.00/0

MOLECULAR PHARMACOLOGY, 40:812-817

cAMP-Dependent Protein Kinase Regulates Inhibition of Adenosine Transport by Ethanol

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Received June 3, 1991; Accepted August 21, 1991

SUMMARY

Ethanol inhibits adenosine uptake, thereby increasing the concentration of extracellular adenosine. Elevation of extracellular adenosine increases intracellular cAMP concentration via activation of adenosine A_2 receptors. Extracellular adenosine is also required for the subsequent development of ethanol-induced heterologous desensitization. Here we report that activation of cAMP-dependent protein kinase is necessary for inhibition of adenosine uptake by ethanol and for the consequent accumulation of extracellular adenosine. Ethanol does not inhibit adenosine uptake in mutants of the S49 cell line that lack receptor-stimulated cAMP production (*unc* cells) or cAMP-dependent protein kinase activity (*kin*$^-$ cells). Forskolin, which bypasses the receptor-coupling defect in *unc* cells to increase cAMP levels, restores inhibition of adenosine uptake by ethanol. In contrast, in *kin*$^-$ cells forskolin did not restore inhibition of adenosine uptake by ethanol, despite similar increases in cAMP levels. Taken together, these results suggest that cAMP-dependent protein kinase phosphorylates a component of the nucleoside transporter, thereby regulating the sensitivity of adenosine transport to ethanol.

The cAMP signal transduction system is a primary target for ethanol in intact cells. Ethanol acutely stimulates cAMP levels (1–6), whereas long term exposure decreases $G_{s\alpha}$ mRNA and protein, leading to decreased cAMP production by receptors coupled to G_s (heterologous desensitization) (5, 7–12). Ethanol-induced receptor desensitization may be of pathophysiologic importance, because circulating lymphocytes (6) and platelet membranes (13) from alcoholic subjects exhibit a similar decrease in receptor-coupled cAMP production.

In several different cell types, acute exposure to ethanol inhibits adenosine uptake via the nucleoside transporter (14, 15). As a result, adenosine accumulates extracellularly and activates adenosine A_2 receptors, increasing intracellular cAMP levels (16). Adenosine accumulation and adenosine receptor activation are also required for chronic ethanol-induced heterologous desensitization of cAMP production (16). After chronic exposure to ethanol, adenosine uptake is no longer inhibited by ethanol and, as a result, accumulation of extracellular adenosine does not occur (14). However, the relationship between ethanol-induced alterations in cAMP levels and the subsequent chronic effects of ethanol on adenosine transport is unclear.

Here we have utilized variants of the S49 cell line to determine whether PKA (EC 2.7.1.37) is required for the inhibition of adenosine uptake by ethanol. We found that ethanol did not inhibit adenosine uptake in cells deficient in cAMP signal transduction. As a result, extracellular adenosine concentrations were not increased by ethanol, and there was no heterologous desensitization after chronic exposure to ethanol. Our data suggest that PKA-mediated phosphorylation of the nucleoside transporter or an associated regulatory component is required for inhibition of adenosine uptake by ethanol, for the accumulation of extracellular adenosine, and for the subsequent development of heterologous desensitization.

Experimental Procedures

Materials. [^{3}H]Adenosine, [^{3}H]uridine, and [^{14}C]methoxyinulin were purchased from Amersham Corp. Chloroacetaldehyde was purchased from Fluka, scintillation fluid from National Diagnostics, mineral oil from Fisher Scientific, and silicone oil from Aldrich. All other reagent-grade chemicals were purchased from Sigma or Boehringer-Mannheim. Cell culture media were purchased from GIBCO.

Cell culture. S49 wild-type and variant cell lines (*unc* and *kin*$^-$) were obtained from the Cell Culture Facility at the University of California, San Francisco. Cells were grown in Dulbecco's modified Eagle medium, with 10% horse serum, and were then transferred to defined medium, containing 1.5 units/ml adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.4) (16), and subcultured every 48 hr for

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ABBREVIATIONS: G_s, stimulatory guanine nucleotide-binding regulatory protein; PKA, cAMP-dependent protein kinase; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIA, phenylisopropyladenosine; PGE_1, prostaglandin E_1; ddFSK, 1,9-dideoxyforskolin.

2–8 weeks. Cells were then seeded at 3.0×10^5 cells/ml, in defined medium without adenosine deaminase, and were cultured for 48 hr in the presence or absence of 100 mM ethanol. Cells were removed from cell culture medium by centrifugation at 200 × *g* for 5 min and were resuspended in assay buffer for specific experiments.

cAMP concentrations. Cells were resuspended at 10^6 cells/ml, in PBS containing 0.2% glucose, 25 mM HEPES, and 1 unit/ml adenosine deaminase, and were preincubated for 5 min at 37° with 10 μM ZK-62711 (a gift from Berlex Labs, Cedar Knolls, NJ), an inhibitor of phosphodiesterase activity. cAMP production was stimulated by incubation of the cells with 100 μM PIA or 1 μM PGE_1 for 15 min, in the presence or absence of 150 mM ethanol (16). Reactions were terminated by addition of 50 μl of 2% Nonidet P-40 in 1 N HCl and incubation of the tubes on ice for 10 min. cAMP levels were determined in a 700 × *g* supernatant by radioimmunoassay (5).

Nucleoside uptake. Cells were resuspended in Hanks' buffered saline containing 25 mM HEPES and were preincubated with 0–200 mM ethanol for 4 min at room temperature (14). Uptake was then determined by addition of 100 μl of the cell suspension to 100 μl of buffered medium containing [^{3}H]adenosine or [^{3}H]uridine (0.01 mCi/ml) and various concentrations of ethanol. After incubation for 60–90 sec, cells were rapidly separated from the medium by centrifugation through 100 μl of inert oil (17). Radioactivity in the cell pellet was measured as previously described (14, 17). Final cell concentrations during the assay were $0.5–1.0 \times 10^7$ cells/ml. Uptake was linear with increasing cell concentrations. Nonspecific uptake of nucleosides was determined after preincubation for 5 min with 10 μM dipyridamole, an inhibitor of nucleoside transport (18). Extracellular fluid in the pellet was measured with [^{14}C]methoxyinulin (19). Ethanol did not alter nonspecific uptake of adenosine or the amount of extracellular fluid in the cell pellet in S49 wild-type and variant cell lines (data not shown).

Enzyme activities. Adenosine deaminase and adenosine kinase (ATP:adenosine-5′-phosphotransferase; EC 2.7.1.20) activities were measured in soluble fractions of wild-type, *unc*, and *kin*$^-$ cells, as previously described (14, 20, 21).

Extracellular adenosine concentration. Cells were suspended at $1–3 \times 10^7$ cells/ml, in PBS prepared with sterile water (16), and were preincubated for 5 min at room temperature. PBS (0.5 ml) containing 0 or 200 mM ethanol was then added to 0.5-ml aliquots of cells. After 10 min, medium was separated by centrifugation of the cells through 100 μl of inert oil. A fluorescent derivative of adenosine in the medium was immediately prepared and separated by reverse phase high pressure liquid chromatography (16, 22). Fluorescence of the eluted sample was monitored on a Kratos FS 970 fluorescence detector, at an excitation wavelength of 280 nm (23, 24), and was compared with that of authentic 1,N^6-ethenoadenosine. Identity of adenosine was confirmed by disappearance of the adenosine peak after pretreatment with adenosine deaminase.

Forskolin treatment. The effect of 10^{-5} to 10^{-7} M forskolin and its inactive derivative ddFSK on adenosine uptake was determined. Cells were incubated with forskolin or ddFSK, or the same concentration of vehicle (33 mM ethanol), for 10 min at 37°, centrifuged for 5 min at 200 × *g*, and resuspended as described above for measurement of nucleoside uptake. cAMP concentration was measured by radioimmunoassay, as described (5).

Statistical analysis. Values are reported as mean ± standard error, unless indicated otherwise. Differences between mean values were analyzed by two-tailed *t* tests and one-way analysis of variance.

Results

Adenosine uptake. To determine whether the cAMP-mediated signal transduction pathway is required for inhibition of adenosine uptake by ethanol, we measured the effects of ethanol on adenosine uptake in wild-type and variant S49 cell lines. The inhibition of [^{3}H]adenosine uptake by ethanol in wild-type cells reached 35% (Fig. 1). In contrast, ethanol did not inhibit uptake in *kin*$^-$ cells, which lack PKA activity, or *unc* cells, in which G_s is present but receptor activation is not coupled to adenylyl cyclase (25) (Fig. 1). The magnitude of adenosine uptake in the absence of ethanol was similar in wild-type, *unc*, and *kin*$^-$ cells (Fig. 1, legend). These results suggest that an active cAMP-dependent phosphorylation pathway is required for inhibition of adenosine uptake by ethanol.

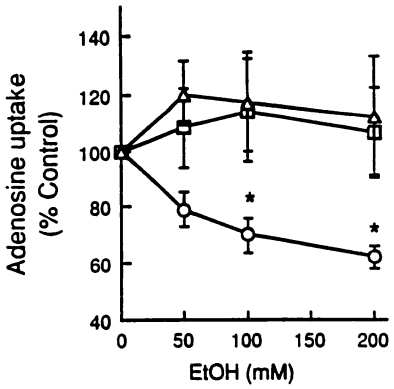

Fig. 1. Ethanol (*EtOH*) inhibition of adenosine uptake in S49 wild-type (○), *kin*$^-$ (□), and *unc* cells (△). Uptake of 0.3 μM [^{3}H]adenosine was measured in the presence of 0–200 mM ethanol at 90 sec. Uptake in the presence of ethanol is expressed as a percentage of uptake in the absence of ethanol. Values represent mean ± standard error of 12 experiments for wild-type cells, three for *kin*$^-$ cells, and four for *unc* cells. Uptake in wild-type, *kin*$^-$, and *unc* cells in the absence of ethanol was 4.38 ± 0.41, 2.47 ± 0.47, and 1.75 ± 0.54 pmol/10^7 cells, respectively. *, $p < 0.05$, compared with cells incubated in the absence of ethanol.

TABLE 1

Adenosine deaminase and adenosine kinase activity in S49 wild-type, *unc*, and *kin*$^-$ cells

Activities of adenosine deaminase and adenosine kinase were measured in S49 wild-type, *unc*, and *kin*$^-$ cells as described in Experimental Procedures. Values represent mean ± standard error of three or four experiments.

Cells	Adenosine deaminase	Adenosine kinase
	nmol/mg of protein/min	*pmol/mg of protein/min*
Wild-type	24.2 ± 5.6	17.1 ± 2.1
unc	34.8 ± 6.6	22.0 ± 3.9
kin$^-$	32.7 ± 6.5	19.4 ± 3.1

[^{3}H]Adenosine uptake is due to influx through the nucleoside transporter, as well as subsequent intracellular metabolism. One or both components may be altered by ethanol. We first confirmed that the differences in sensitivity of adenosine uptake to ethanol between wild-type and mutant cells were not due to differences in adenosine metabolism. No difference was found in activity of the primary adenosine-metabolizing enzymes, adenosine deaminase or adenosine kinase, between wild-type, *kin*$^-$, and *unc* cells (Table 1). We next determined the effect of ethanol on the uptake of uridine, another nucleoside transported by the nucleoside transporter but metabolized differently than adenosine. Uptake of [^{3}H]uridine was inhibited by ethanol in wild-type cells (Fig. 2). If cAMP-dependent phosphorylation at the level of nucleoside transport is required for inhibition of adenosine uptake by ethanol, then uptake of uridine in *kin*$^-$ and *unc* cells should also be unaffected by ethanol. Similar to the results obtained with adenosine (Fig. 1), ethanol did not inhibit [^{3}H]uridine uptake in *unc* or *kin*$^-$ cells (Fig. 2). Taken together, these results suggest that inhibition of adenosine uptake by ethanol is regulated by cAMP at

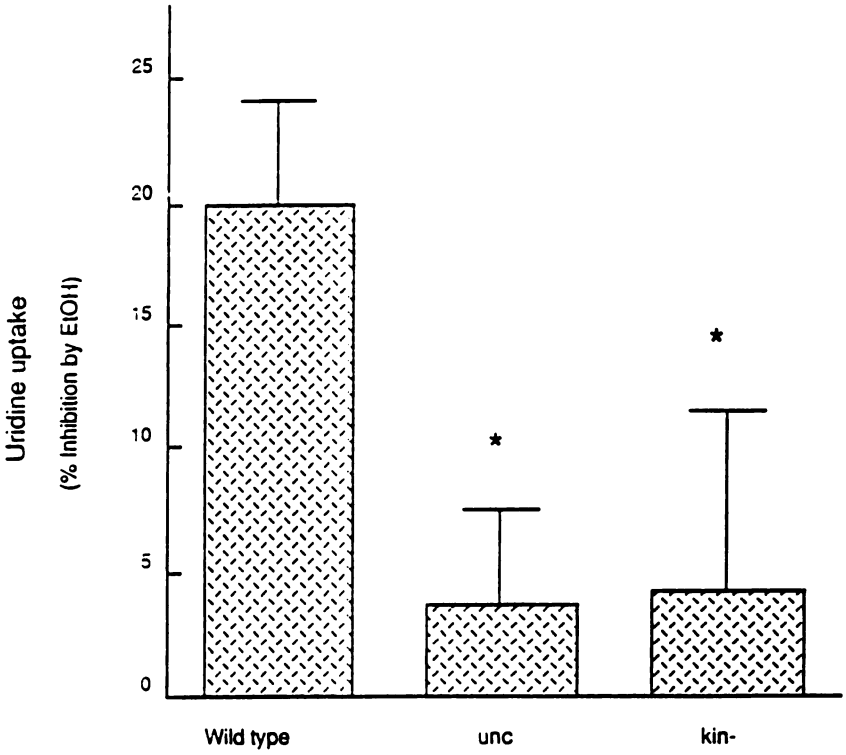

Fig. 2. Inhibition of uridine uptake by ethanol in S49 wild-type, *kin*$^-$, and *unc* cells. Uptake of 0.14 μM [^{3}H] uridine was measured at 60 sec, in the presence or absence of 100 mM ethanol. Percentage of inhibition by ethanol is expressed in relation to cells incubated in the absence of ethanol. Uptake in wild-type, *kin*$^-$, and *unc* cells in the absence of ethanol was 0.45 ± 0.04, 0.6 ± 0.1, and 0.7 ± 0.2 pmol/10^7 cells, respectively. Values represent mean ± standard error of six to eight experiments. *, $p < 0.05$, compared with wild-type cells.

the level of nucleoside transport (26) and is not due to changes in adenosine metabolism.

Decreased intracellular cAMP and consequently decreased PKA in *unc* cells and the lack of PKA activity in *kin*$^-$ cells should result in decreased cAMP-dependent protein phosphorylation (27). If adenosine uptake in *unc* cells is insensitive to ethanol because of decreased phosphorylation, then forskolin, which increases cAMP concentration independently of receptor activation (28), should restore inhibition of adenosine uptake by ethanol. When adenosine uptake was measured in *unc* cells that had been treated with increasing concentrations of forskolin for 10 min, inhibition of adenosine uptake by ethanol was restored (Fig. 3). ddFSK, a forskolin derivative that does not increase cAMP, did not restore ethanol sensitivity (data not shown), suggesting a specific effect of forskolin on cAMP generation. Forskolin failed to restore sensitivity to ethanol in *kin*$^-$ cells (Fig. 4), which lack PKA activity, despite increases in cAMP levels. Because forskolin had no effect on adenosine uptake in the absence of ethanol in all three cell types (Fig. 4), these results suggest that cAMP-dependent phosphorylation is required only for inhibition of adenosine uptake by ethanol.

Extracellular adenosine concentrations. In wild-type S49 cells, acute exposure to ethanol results in an increase in extracellular adenosine concentration, which we postulated is due to inhibition of adenosine uptake by ethanol (16). Because ethanol did not inhibit adenosine uptake in *kin*$^-$ cells, these cells should not accumulate extracellular adenosine on exposure to ethanol. When wild-type cells were incubated in 100 mM ethanol for 10 min, extracellular adenosine concentrations increased 1.8-fold (Fig. 5) (16). In contrast, ethanol did not increase extracellular adenosine concentrations in *kin*$^-$ cells (Fig. 5). These results suggest that cAMP-dependent phosphorylation, which is required for inhibition of adenosine transport by ethanol, regulates ethanol-induced increases in extracellular adenosine.

Ethanol-induced heterologous desensitization. Extracellular adenosine is required for ethanol-induced heterologous desensitization in S49 wild-type cells (14). In *kin*$^-$ cells, ethanol did not inhibit adenosine uptake and, consequently, did not increase extracellular adenosine concentrations. Therefore, chronic exposure to ethanol should not lead to heterologous desensitization of receptor-dependent cAMP production in these cells. After chronic exposure of S49 wild-type cells to 100 mM ethanol for 48 hr, PIA and PGE_1 receptor-stimulated cAMP levels were decreased by 28% and 36%, respectively (Fig. 6). In contrast, receptor-dependent cAMP production was not decreased in *kin*$^-$ cells after the same exposure to ethanol (Fig. 6), suggesting that PKA is required for ethanol-induced heterologous desensitization.

Discussion

Inhibition of adenosine transport by ethanol mediates acute ethanol-induced increases in cAMP levels and is required for the subsequent development of heterologous desensitization (16). Acute exposure to ethanol inhibits adenosine uptake, leading to an increase in extracellular adenosine concentration (14). Adenosine then activates adenosine A_2 receptors to increase intracellular cAMP (16). Upon continued exposure to ethanol, there is a heterologous desensitization of receptors coupled to adenylyl cyclase via G_s, leading to decreased cAMP levels (16), and adenosine uptake is no longer inhibited by ethanol (14).

These observations suggest a relationship between the effects of ethanol on the cAMP signal transduction system and inhibition of adenosine uptake by ethanol. Therefore, we examined inhibition of adenosine uptake by ethanol in variants of the S49 cell line that are deficient in receptor-dependent cAMP production (*unc*) and PKA activity (*kin*$^-$). In S49 wild-type cells, acute exposure to ethanol inhibited adenosine uptake,

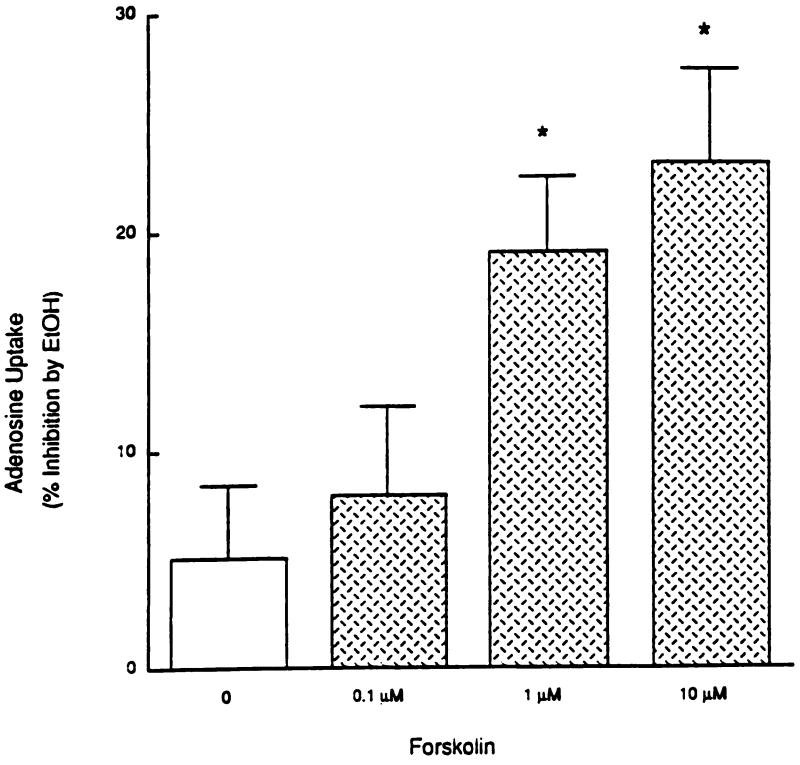

Fig. 3. Inhibition of adenosine uptake by ethanol in *unc* cells as a function of forskolin concentration. Uptake of 0.14 μM [^{3}H]adenosine was measured at 60 sec, in the presence or absence of 200 mM ethanol, after preincubation of *unc* cells with or without forskolin at the indicated concentrations. Percentage of inhibition by ethanol is expressed as in Fig. 2. Values represent mean ± standard error of four to eight experiments. *, $p < 0.008$, compared with cells not treated with forskolin.

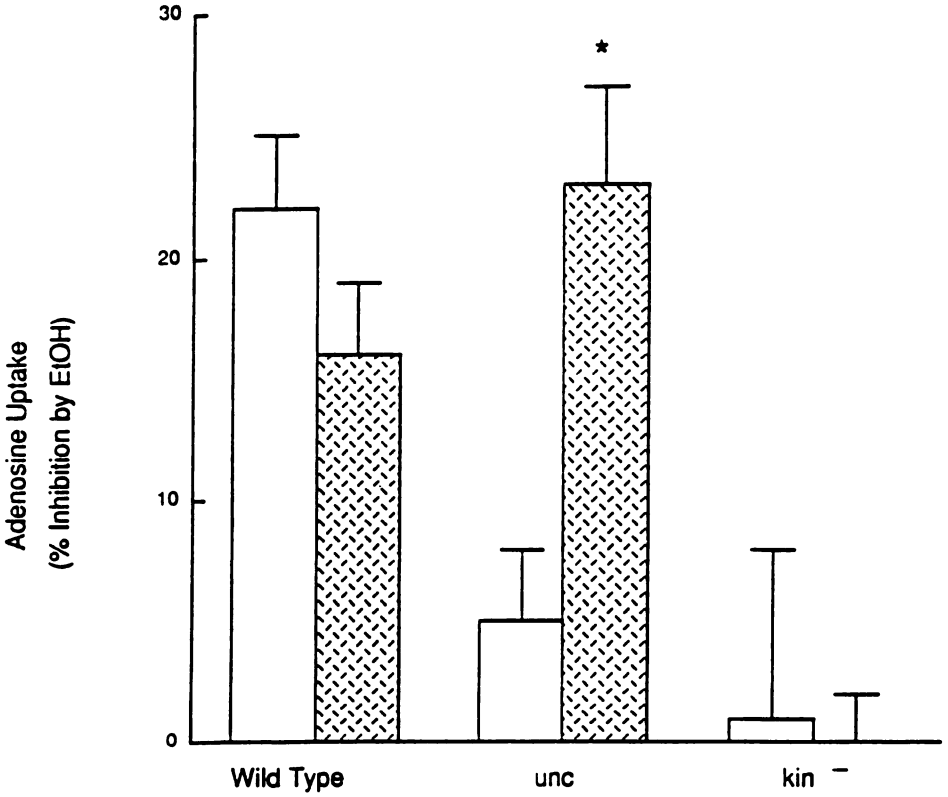

Fig. 4. Inhibition of adenosine uptake by 200 mM ethanol in wild-type, *unc*, and *kin*$^-$ cells after treatment with forskolin. Cells were treated with (▨) or without (□) 10^{-5} M forskolin, and adenosine uptake was measured as described in Fig. 3. Forskolin had no effect on adenosine uptake in the absence of ethanol (uptake after forskolin treatment was 95 ± 5% of uptake in cells in the absence of forskolin; 17 experiments). Forskolin-stimulated cAMP levels did not differ between cell types (1030 ± 160 pmol/10^7 cells; seven experiments). Values represent mean ± standard error of three to eight experiments. *, $p < 0.02$, compared with cells not treated with forskolin.

causing increased extracellular adenosine concentrations (14). In contrast, ethanol did not inhibit adenosine uptake in *kin*$^-$ or *unc* cells (Fig. 1). Consequently, ethanol did not increase extracellular adenosine (Fig. 5) and chronic ethanol-induced heterologous desensitization did not occur (Fig. 6) in *kin*$^-$ cells. In *unc* cells, inhibition of adenosine uptake by ethanol could be restored by raising of cAMP levels with forskolin (Figs. 3 and 4). Increasing of cAMP levels in *kin*$^-$ cells by forskolin had no effect (Fig. 4). Because *kin*$^-$ cells lack PKA activity, these results suggest that cAMP-dependent phosphorylation is required for ethanol to inhibit adenosine uptake.

Adenosine uptake is due to both influx of adenosine via the nucleoside transporter and subsequent intracellular metabolism. Our data suggest that ethanol sensitivity of nucleoside

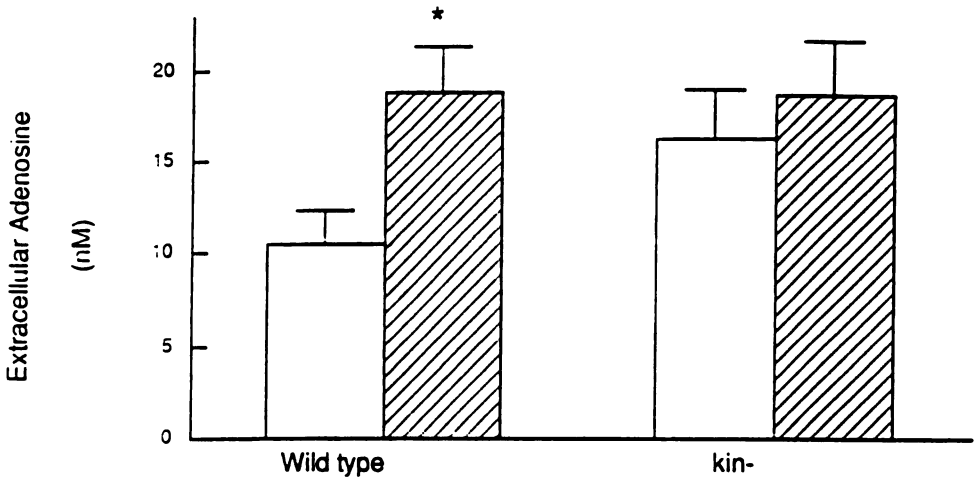

Fig. 5. Extracellular adenosine concentrations in S49 wild-type and *kin*⁻ cells after acute exposure to ethanol. Cells were incubated with (▨) or without (□) 100 mM ethanol for 10 min. The extracellular adenosine concentration was determined by high pressure liquid chromatography, as described in Experimental Procedures. Values represent mean ± standard error of nine experiments for wild-type cells and four experiments for *kin*⁻ cells. *, $p < 0.003$, compared with cells not exposed to ethanol.

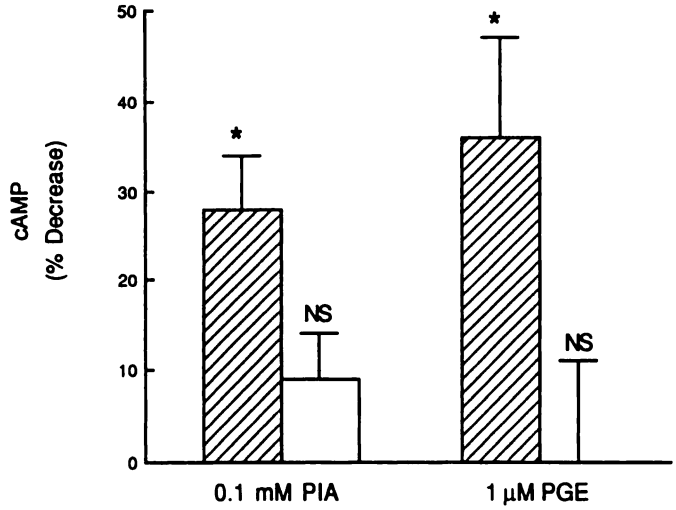

Fig. 6. Receptor-stimulated cAMP levels in S49 wild-type (▨) and *kin*⁻ cells (□) produced by incubation for 48 hr with 100 mM ethanol. Results are expressed as the percentage decrease of PIA or PGE_1 stimulation in chronic ethanol-treated cells, compared with control cells grown in the absence of ethanol. *, $p < 0.007$, compared with controls. PIA-stimulated cAMP levels in cells not treated with ethanol were 2.0 ± 0.2 pmol/10^6 cells (130% stimulation over basal) for wild-type cells and 36 ± 5 pmol/10^6 cells (119% stimulation) for *kin*⁻ cells. PGE_1-stimulated levels in wild-type and *kin*⁻ cells were 7.7 ± 0.5 (402% stimulation) and 95 ± 7 pmol/10^6 cells (290% stimulation), respectively. Values are mean ± standard error of four or five experiments. *NS*, not significant.

transport, rather than intracellular metabolism, is regulated by phosphorylation. Adenosine and uridine are both transported by the nucleoside transporter but are metabolized by different intracellular pathways (26, 29–33). We found that ethanol inhibited both uridine and adenosine uptake in wild-type cells but had no effect on the uptake of either of these nucleosides in *unc* or *kin*⁻ cells (Figs. 1 and 2). Adenosine deaminase, which deaminates adenosine to inosine, and adenosine kinase, which phosphorylates adenosine to AMP, are primarily responsible for adenosine metabolism (26). Neither acute nor chronic exposure to ethanol alters activity of these enzymes in wild-type cells (14). Moreover, we were unable to demonstrate any differences in enzyme activities in the variant cell lines, compared with the wild-type (Table 1). Taken together, these data suggest that a common ethanol-sensitive component of the transport system, either the nucleoside transporter itself or an associated regulatory component, is regulated by phosphorylation. This phosphorylation is required for ethanol to inhibit adenosine uptake. Confirmation of such a regulatory mechanism awaits purification of the nucleoside transporter.

Although it has been proposed that the nucleoside transporter may be allosterically regulated (34, 35), there are conflicting reports on regulation of nucleoside transport by cAMP (26, 36, 37). Early studies suggested that decreases in cAMP produced by serum or insulin increased uridine uptake (36, 37). In those experiments, a reduction in cAMP levels preceded increases in uridine uptake (36). However, later studies showed no alteration in initial uptake rates due to serum- or insulin-induced changes in cAMP (26). Subsequently, Wohlheuter *et al.* (38) reported that increased cAMP levels had no effect on transport of adenosine or uridine in Chinese hamster ovary cells. Consistent with these latter observations, increasing of cAMP levels with forskolin had no effect on adenosine uptake in the absence of ethanol in S49 wild-type, *unc*, or *kin*⁻ cells (see legend to Fig. 4). cAMP-dependent phosphorylation appears to be required only for sensitivity to ethanol.

After prolonged exposure of S49 wild-type cells to ethanol, adenosine uptake is no longer inhibited by rechallenge with ethanol (14), i.e., nucleoside transport has lost its sensitivity to ethanol. Because this insensitivity is similar to that found in *unc* and *kin*⁻ cells, the insensitivity in cells chronically exposed to ethanol might be due to decreased cAMP levels (5, 7–12) and, presumably, decreased phosphorylation of the nucleoside transport system.

The role of phosphorylation in the sensitivity of adenosine uptake to ethanol is unknown. Because ethanol binds to hydrophobic moieties on proteins (39–43), ethanol might bind directly to the transporter, causing a conformational change that would result in inhibition of uptake. Our data suggest that the adenosine transport system has to be "primed" by phosphorylation before ethanol exposure, i.e., ethanol would only bind to the transporter and/or inhibit uptake if the nucleoside transporter or an associated regulatory component were phosphorylated.

In summary, the findings in this study suggest that, in S49 cells, cAMP-dependent phosphorylation of the nucleoside transporter is required for acute inhibition of adenosine uptake by ethanol, the accumulation of extracellular adenosine, and the subsequent development of ethanol-induced heterologous

desensitization. These cells adapt to ethanol by lowering cAMP levels, which may then result in an insensitivity of the adenosine transporter to the inhibitory effects of ethanol. Because ethanol has similar effects on the adenosine transporter and cAMP signal transduction in a neural cell line (16) and in human peripheral lymphocytes (8),[2] regulation of the sensitivity of adenosine transport to ethanol by cAMP-dependent phosphorylation may be important in the pathophysiology of alcoholism.

Acknowledgments

We thank Christopher Franklin, David Casso, and Lelen Lopez for their technical assistance.

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