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Inhibition of Adenosine Uptake by Ethanol Is Specific for One Class of Nucleoside Transporters

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SUMMARY

Adenosine uptake via nucleoside transporters is inhibited when S49 and NG108-15 cell lines cells are exposed to ethanol. This inhibition leads to an accumulation of extracellular adenosine that binds to adenosine A2 receptors and increases cAMP production. Subsequently, there is a heterologous desensitization of receptors coupled to adenylyl cyclase for which adenosine also is required. There are multiple classes of facilitative and concentrative nucleoside transporters that could be inhibited by ethanol to initiate this cascade of events. In this paper, we establish that adenosine uptake by only one type of nucleoside transporter, an NBMPR-sensitive facilitative transporter, is inhibited by ethanol. There is no effect on other classes of nucleoside transporters even when present in the same cell. Thus, ethanol-induced extracellular accumulation of adenosine results specifically from inhibition of NBMPR-sensitive facilitative nucleoside transporters. We also find that human lymphocytes express only facilitative nucleoside transporters and that the NBMPR-sensitive type is predominant. Thus, inhibition of this type of transporter by ethanol may be related to the desensitization of cAMP signal transduction that we have reported in lymphocytes from alcoholics.

Extracellular adenosine can produce diverse physiologic effects by binding to A1 or A2 adenosine receptors that activate intracellular signal transduction systems (1–3). Effects of adenosine include inhibition of excitatory neurotransmitter release, regulation of immune function, reduction of cardiac contractility, vasodilation, inhibition of platelet aggregation, and lipolysis (3–6).

Nucleoside transporters mediate passage of adenosine and other nucleosides across the plasma membrane of mammalian cells. Blockers of adenosine transport potentiate cellular responses to adenosine, which suggests that the physiologic effects of adenosine are terminated in part by re-uptake of adenosine into the cell (7–9). At least four classes of mammalian nucleoside transporters have been identified: two that are bidirectional facilitative transporters and two that are unidirectional concentrative transporters (10–12).

Recent evidence indicates that adenosine mediates many behavioral effects of ethanol in experimental animals (13–15). In addition, adenosine appears to mediate ethanol-induced hyperpolarization of hippocampal dentate granule neurons (16). We reported that extracellular adenosine is required for acute and chronic ethanol-induced changes in cAMP signal transduction in S49 and NG108 cell culture systems (17, 18). Accumulation of extracellular adenosine results when ethanol inhibits adenosine uptake in many cultured cells (19, 20). The accumulated adenosine then activates adenosine A2 receptors to stimulate cAMP production. Continued exposure to ethanol leads to heterologous desensitization of receptors coupled to G_s. This decrease in receptor-stimulated cAMP levels is correlated with decreased mRNA, protein, and activity of the α-subunit of G_s (21). Thus, our results indicate that inhibition of adenosine uptake by ethanol leads to heterologous desensitization of cAMP signal transduction, which would have pleiotropic effects on cells.

Circulating lymphocytes from chronic alcoholics also exhibit desensitization of receptor-stimulated cAMP production. There is a 76% reduction in cAMP levels in freshly isolated lymphocytes from actively drinking alcoholics when compared with control subjects (22). By analogy to our cell culture model, ethanol inhibition of nucleoside transporters in human lymphocytes could be the initial event leading to subsequent desensitization of cAMP signal transduction in lymphocytes from alcoholics. We report here that human lymphocytes express facilitative but not concentrative nucleoside transporters and the predominant transporter is sensitive to NBMPR. Furthermore, we find that ethanol inhibits only NBMPR-sensitive facilitative nucleoside transporters.

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ABBREVIATIONS: G_s, stimulatory guanine nucleotide protein; NBMPR, nitrobenzylmercaptopurine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials and Methods

Chemicals and reagents. [^{3}H]Formycin (7–14 Ci/mm) was purchased from Moravek Biochemicals, Inc. (Brea, CA), and [^{3}H]glutamine (53 Ci/mm), [^{3}H]isoleucine (92 Ci/mm), and [^{3}H]thymidine (45 Ci/mm) were from Amersham Intl. (Buckinghamshire, UK). Formycin B was from Calbiochem Novabiochem Corp. (La Jolla, CA), scintillation fluid from National Diagnostics Inc. (Somerville, NJ), mineral oil from Fisher Scientific (Pittsburgh, PA), silicone oil from Aldrich Chemical Co. (Milwaukee, WI), and cell culture media from GIBCO BRL (Gaithersburg, MD). All other reagent-grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer Mannheim (Indianapolis, IN).

Cells. Peripheral blood was collected from healthy male volunteers (ages 25–55 yr) into heparinized vacutainers and the isolated lymphocytes were either used immediately ("circulating" lymphocytes) or seeded at 1 to 1.5 × 10^6 cells/ml in L2 medium and cultured for 7 days at 37° in humidified 5% CO_2/95% air as described previously (23). Wild-type L1210 murine lymphoma cells and the L1210/MA27.1 mutants were provided generously by Dr. Judith A. Belt and grown in RPMI 1640 medium supplemented with 10% refiltered, heat-inactivated horse serum. N1S1 hepatoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 with 10% fetal calf serum. Cells were adapted gradually to growth in L2 medium and were maintained at least 4 days before use for acute ethanol inhibition assays. Cell growth and viability were determined by cell counting and trypan blue exclusion.

Formycin uptake. Cells were centrifuged and the pellet resuspended at 2–8 × 10^7 cells/ml in Hanks balanced salt solution containing 25 mM HEPES and 0.5 mM dithiothreitol. For experiments in other sodium-containing and sodium-free media, the initial cell pellet was resuspended in the appropriate medium, and the cells were recollected and resuspended in the corresponding media. Effects of ethanol, NBMPR, or dipyridamole were determined after preincubating the cells for 4 min at room temperature. For ethanol exposure, a concentration of 200 mM was used routinely, although significant inhibition can be detected at lower concentrations (19, 24, 25). Uptake (in 200 μl) was initiated by addition of cells to [^{3}H]formycin (5 μM; 1.5 Ci/mm). After incubation at room temperature, the reactions were terminated by rapid centrifugation of the cells through mineral-silicone oil (12:88) or by addition of 50 μl of 100 μM ice-cold dipyridamole followed by sedimentation through oil. After removal of the oil, radioactivity was determined as described previously (19). All assays were performed in triplicate.

Glutamine and isoleucine uptake. Transport was measured as described (19, 26) with some modifications. Briefly, cultured lymphocytes were harvested by centrifugation, resuspended at 5–7 × 10^7 cells/ml in Hanks balanced salt solution containing 25 mM HEPES with 0.5 mM dithiothreitol, and preincubated for 4 min at room temperature in the presence or absence of 200 mM ethanol. Transport was measured with 2 μCi of either [^{3}H]glutamine (0.4 mM) or isoleucine (50 μM). Glutamine transport was terminated at 1 min by the addition of ice-cold 160 mM unlabeled glutamine; isoleucine transport was terminated by 20 mM unlabeled isoleucine at 90 sec. Nonspecific binding was determined using either 40 mM unlabeled glutamine or 5 mM unlabeled isoleucine. Assays for ethanol inhibition of amino acid uptake always were performed in parallel with assays for ethanol inhibition of formycin uptake. All assays were performed in triplicate.

Results

Nucleoside transport in human lymphocytes

There are multiple distinct facilitative and concentrative nucleoside transport systems that mediate passage of adenosine and other nucleosides across mammalian cell membranes (10–12). Two classes of sodium-independent facilitative membrane transporters accept ribosides, deoxyribosides, and their analogs. One type has high affinity binding sites for NBMPR which is inhibitory ("NBMPR-sensitive" transporters), whereas the other type lacks NBMPR binding sites and is relatively NBMPR insensitive ("NBMPR-resistant" transporters). Both of these facilitative nucleoside transport systems can be inhibited by dipyridamole. In contrast, two sodium-dependent concentrative nucleoside transporters are relatively insensitive to both dipyridamole and NBMPR but differ in substrate specificity.

We have characterized the nucleoside transporter classes present on human lymphocytes using formycin, a nucleoside analog of inosine. Because formycin is not metabolized in human lymphocytes,[2] it can be used to evaluate nucleoside transport processes independent of metabolism (27).

Facilitative transport. Uptake of formycin by sodium-independent facilitative transporters was measured in cultured human lymphocytes in a sodium-free medium. Initially, formycin uptake increases linearly with time but reaches a plateau at 20 sec (Fig. 1A). Uptake is reduced by dipyridamole (Fig. 1A) to a level comparable with that of nonspecific binding,

[2] S. W. Krauss, unpublished observations.

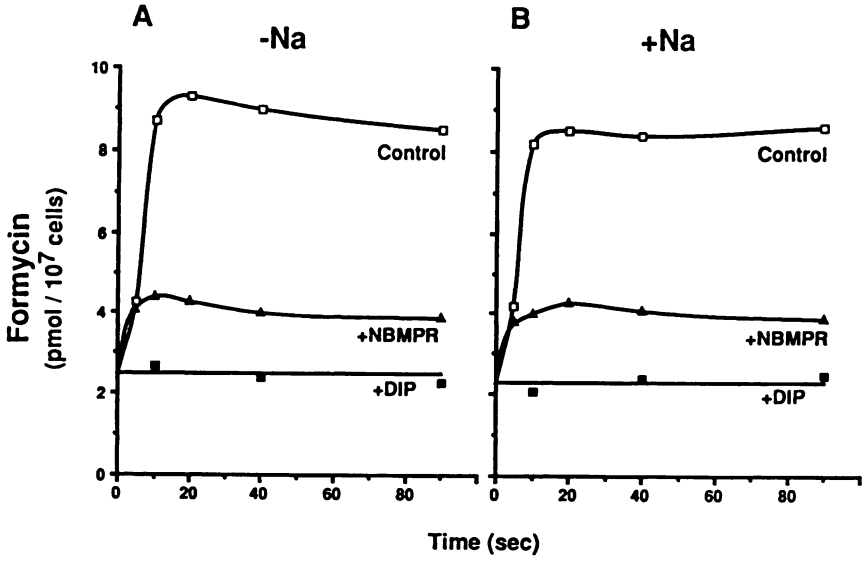

Fig. 1. Time course for formycin uptake by cultured human lymphocytes in the presence or absence of sodium. Uptake of [^{3}H]formycin was assayed as described in Materials and Methods in 5 mM Tris (pH 7.5), 10 mM glucose, 1 mM $MgCl_2$, and either 145 mM choline chloride (sodium-free medium) or 145 mM NaCl (sodium medium), at the indicated times without additions (control), with 1 μM NBMPR (+ NBMPR), or with 10 μM dipyridamole (+ *DIP*). Values are the mean ± standard deviation of triplicate determinations.

determined by measuring uptake in the presence of excess unlabeled formycin (data not shown). NBMPR also decreases formycin uptake, but not to the same extent as dipyridamole. Thus, cultured human lymphocytes possess both NBMPR-sensitive and NBMPR-resistant nucleoside uptake activities, which are characteristic of facilitative transporters. We also analyzed formycin uptake in freshly isolated circulating human lymphocytes. Cultured and circulating lymphocytes, examined under linear uptake conditions, exhibit predominantly NBMPR-sensitive formycin uptake (71% and 95%, respectively) (Table 1).

Concentrative sodium-dependent transport. Formycin uptake in cultured human lymphocytes in a sodium-containing medium is similar to uptake in the absence of sodium. Uptake is inhibited by both dipyridamole and NBMPR (Fig. 1B). The small but detectable amount of NBMPR-resistant uptake does not continue to increase with time.

Concentrative uptake was also assayed in the presence or absence of dipyridamole after 60 min of incubation at 37° in sodium-containing medium (Table 2). These were the same conditions previously used to assay sodium-dependent concentrative transport in a variety of murine cell lines (28). Two well characterized murine lymphoma lines were used as positive controls for detection of concentrative transport in our experiments: L1210 wild type cells which have facilitative uptake as well as concentrative formycin uptake (29), and MA27.1, an L1210 mutant line which has only sodium-dependent concentrative formycin uptake (30). L1210 wild type cells take up more formycin in the presence of dipyridamole than in its absence, probably because dipyridamole blocks efflux via facilitative transporters. Dipyridamole does not affect formycin transport in MA27.1 cells that contain only concentrative transporters. By contrast, formycin uptake in both cultured and circulating human lymphocytes is completely inhibited by dipyridamole in both sodium-containing (Table 2) and sodium-free medium (data not shown). There also was no evidence in circulating or cultured human lymphocytes of sodium-dependent concentrative uptake of thymidine (data not shown), another model substrate for concentrative transport (31). These results indicate that cultured human lymphocytes do not have sodium-dependent concentrative nucleoside uptake; human lymphocytes possess only sodium-independent NBMPR-sensitive and NBMPR-resistant facilitative nucleoside uptake systems.

TABLE 1

NBMPR inhibits formycin uptake in cultured and circulating human lymphocytes

Uptake of [^{3}H]formycin in Hanks' balanced salt solution containing HEPES was determined in the absence or presence of 1 μM NBMPR for 12 sec with cultured cells (linear range extends to 20 sec) or 30 sec with circulating lymphocytes (linear range extends to 60 sec) as described in Materials and Methods. Nonspecific uptake in the presence of 10 μM dipyridamole was subtracted, and values represent the mean ± standard error for n = 8 individuals (23 determinations) for cultured and n = 6 for circulating lymphocytes.

Cell type	Formycin uptake		Inhibition
	−NBMPR	+NBMPR	
	pmol/10^7 cells		%
Cultured lymphocytes	6.1 ± 0.4	1.8 ± 0.1	71
Circulating lymphocytes	0.91 ± 0.2	0.05 ± 0.02	95

TABLE 2

Nucleoside uptake in the presence of sodium

[^{3}H]Formycin uptake was determined in triplicate in the absence or presence of 10 μM dipyridamole for 60 min at 37° in a buffer containing 120 mM NaCl, 20 mM Tris, pH 7.4, 3 mM potassium phosphate, 1 mM $MgCl_2$, and 10 mM glucose. Nonspecific uptake measured with 5 mM unlabeled formycin was subtracted for L1210 and MA27.1 cells. In the case of human lymphocytes, the average values with unlabeled formycin were not statistically different from those presented for dipyridamole. The values are the mean ± standard error of three independent experiments (n = 2 for L1210).

Cell type	−Dipyridamole	+Dipyridamole
Cultured lymphocytes	18.1 ± 5.1	8.5 ± 2.6
Circulating lymphocytes	3.1 ± 0.6	1.4 ± 0.2
L1210	9.5 ± 2.0	180 ± 38
MA27.1	477 ± 47	461 ± 68

Ethanol sensitivity of nucleoside uptake in human lymphocytes

In previous studies we established that adenosine transport in cultured cell lines (19, 24) and human lymphocytes (25) is inhibited by a wide range of ethanol concentrations. When human lymphocytes were tested, we found that 200 mM ethanol inhibited formycin uptake by 38% ($p < 0.005$) in cultured human lymphocytes and by 40% ($p < 0.001$) in circulating human lymphocytes (Fig. 2). In contrast, uptake of isoleucine and glutamine in these cells is not affected by ethanol treatment (Fig. 2). Thus, ethanol inhibition of nucleoside uptake in human lymphocytes is not because of a global effect on all membrane transport systems.

Specificity of ethanol inhibition of nucleoside uptake

Because human lymphocytes contain only NBMPR-sensitive and NBMPR-resistant facilitative nucleoside transporters, one or both of these systems could be ethanol sensitive. When cultured lymphocytes are exposed to ethanol, formycin uptake is inhibited by 36% ($p < 0.005$) in cultured human lymphocytes,

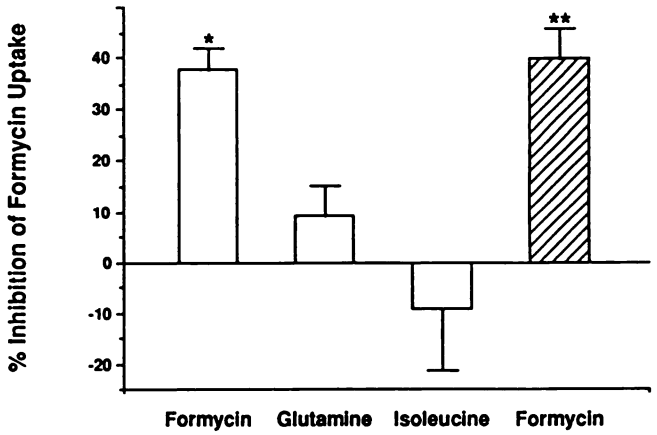

Fig. 2. Effects of ethanol on formycin, glutamine, and isoleucine uptake in human lymphocytes. Uptake of 5 μM [^{3}H]formycin, 400 μM [^{3}H]glutamine, or 50 μM [^{3}H]isoleucine was measured after a 4-min preincubation with or without 200 mM ethanol. Nonspecific uptake (10 μM dipyridamole for formycin or 100-fold unlabeled amino acid) was subtracted from total uptake. Assays of amino acid uptake always were conducted in parallel with assays of formycin uptake. All assays were performed in the linear range of uptake. *Open bars* represent measurements in cultured human lymphocytes; the *striped bar* indicates ethanol inhibition of formycin in circulating human lymphocytes. The results are normalized to uptake in the absence of ethanol (6.2 ± 0.3 pmol/10^7 cells for formycin in cultured lymphocytes, 0.8 ± 0.2 pmol/10^7 cells of formycin in circulating lymphocytes, 20.0 ± 6.6 pmol/10^6 cells for glutamine, and 26 ± 1.5 pmol/10^6 cells for isoleucine). The *error bars* indicate standard error for n = 10 (formycin, cultured), n = 6 (formycin, circulating), n = 5 (glutamine), and n = 3 (isoleucine) independent experiments. * $p < 0.005$ and ** $p < 0.001$ compared with respective controls.

but NBMPR-resistant transport is not affected by ethanol within the limits of detection of the assay (Fig. 3). Similar results were obtained for L1210 cells when assayed in sodium-free medium to minimize the contribution of their concentrative transporters: ethanol inhibits nucleoside uptake (30%; $p < 0.03$) but has no significant effect on NBMPR-resistant uptake (Fig. 3).

Because NBMPR-resistant uptake is low in human lymphocytes and L1210 cells, we performed experiments using N1S1 hepatoma cells to increase the sensitivity of detection of the effect of ethanol on NBMPR-resistant uptake. In N1S1 cells, 80% of formycin uptake is NBMPR-resistant. There is a small inhibition (15%; $p < 0.02$) of nucleoside uptake by ethanol in the absence of NBMPR, probably reflecting the NBMPR-sensitive uptake (20%) detected in these cells. However, there is no further inhibition of nucleoside uptake by ethanol in the presence of NBMPR (Fig. 3), consistent with the results in lymphocytes and L1210 cells.

MA27.1 cells were used to determine if ethanol affects concentrative nucleoside uptake. Formycin uptake in these cells remains unchanged after ethanol exposure (Fig. 3). Because MA27.1 cells express only concentrative transporters, this class of transporters is not inhibited by ethanol.

In summary, our results demonstrate that ethanol does not inhibit formycin uptake significantly by either NBMPR-resistant or concentrative transport systems. Ethanol inhibition of nucleoside uptake is specific for only NBMPR-sensitive facilitative nucleoside transporters.

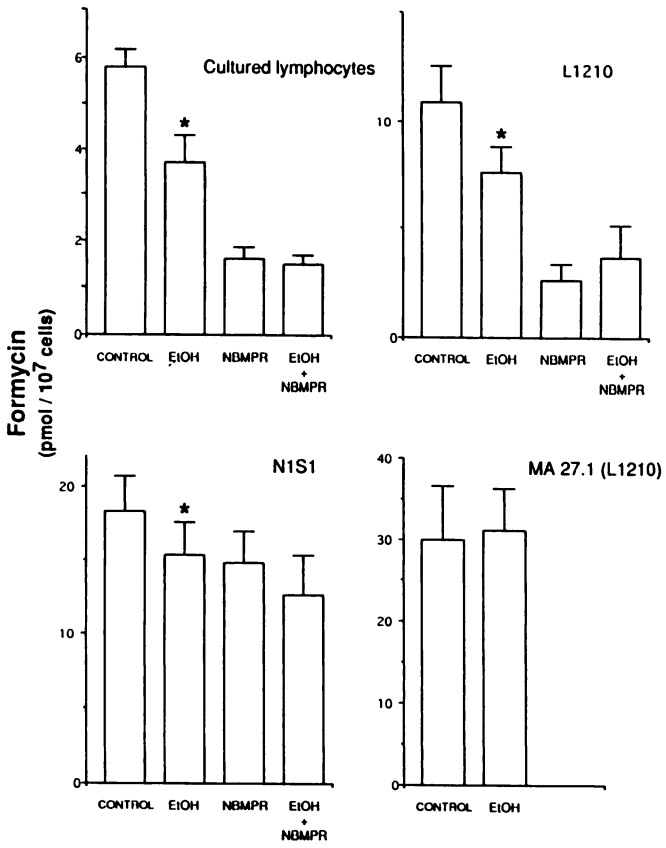

Fig. 3. Effect of ethanol on facilitative and concentrative nucleoside transporters in various cell types. [^{3}H]Formycin uptake was measured for 12 sec (cultured human lymphocytes, L1210, and N1S1 cells) or 1 min for MA27.1 cells. Uptake was within the linear range in all cases (data not shown). Assays in triplicate were performed in the absence or presence of 200 mM ethanol, 1 μM NBMPR, or 200 mM ethanol and 1 μM NBMPR as described in Materials and Methods. Experiments with MA27.1 contained 10 μM dipyridamole. Values are corrected for nonspecific uptake with 10 μM dipyridamole or 5 mM unlabeled formycin (MA27.1). The experiments with human lymphocytes were conducted independently of those presented in Fig. 2. Values represent the mean ± standard error. *P* values are compared with controls: human lymphocytes, $n = 3$, $^*p < 0.005$; L1210, $n = 4$, $^*p < 0.03$; N1S1, $n = 4$, $^*p < 0.02$; MA27.1, $n = 4$.

Discussion

Almost all cells have nucleoside transporters, but the classes expressed vary with species and cell type. Mammalian cells usually express facilitative nucleoside transporters, but the proportion of NBMPR-sensitive versus NBMPR-resistant transport varies. For example, NBMPR inhibits uptake by 50% in a human HeLa line (32), whereas uptake is completely inhibited by NBMPR in human erythrocytes (33, 34). In our study, we find that formycin uptake in both circulating and cultured lymphocytes is predominantly NBMPR-sensitive (Table 1), but is about 6-fold greater in cultured lymphocytes than in circulating lymphocytes. Because of the importance of nucleoside uptake for DNA synthesis, this difference may reflect the fact that cultured cells are actively dividing while circulating lymphocytes are not.

In addition to facilitative nucleoside uptake, there are at least two kinds of sodium-dependent concentrative nucleoside transporters that are differentially expressed in different cell types (35, 36). Both types of concentrative transporters are relatively insensitive to dipyridamole and NBMPR, but they have distinct substrate specificities. One type accumulates formycin but not thymidine, whereas the other does the converse (36). We did not detect concentrative uptake of formycin or thymidine in human lymphocytes even when facilitative transport is abolished (Table 2). Therefore, our studies show that human lymphocytes express only facilitative nucleoside transporters that are predominantly NBMPR-sensitive. There is only one other report measuring nucleoside uptake in nontransformed nucleated human cells (37). However, formycin uptake values in these cells (human macrophages) were so low that dipyridamole effects are difficult to interpret. NBMPR sensitivity was not tested.

A major finding of our study is that only NBMPR-sensitive nucleoside transporters are inhibited by ethanol. This is a very specific effect because other nucleoside (Fig. 3) and amino acid transporters (Fig. 2) are unaffected by acute treatment with ethanol. Furthermore, the specificity of ethanol inhibition is independent of cell type. Ethanol inhibits nucleoside uptake in cells expressing NBMPR-sensitive transporters such as human lymphocytes (Fig. 3), L1210 (Fig. 3), NG108-15,[3] S49 (18) HepG2[2], CHO[2], and hepatocytes (38). Ethanol has also been shown to inhibit adenosine uptake in synaptosomal fractions of rat brain (39). This inhibition is presumably the result of the presence of NBMPR-sensitive transporters in synaptosomes (40).

We have shown that the NBMPR-sensitive nucleoside transporter is required for ethanol-induced desensitization; desensitization did not occur in S49 mutants lacking nucleoside transport. The molecular events that regulate ethanol inhibition of NBMPR-sensitive nucleoside uptake are unknown.

[3] Sapru, M., I. Diamond and A. S. Gordon, manuscript submitted.

However, phosphorylation by cAMP-dependent protein kinase A appears to be required because nucleoside uptake in wild type S49 cells is inhibited by ethanol; but there is no inhibition in S49 mutants lacking either receptor-stimulated cAMP production (*unc* cells) or cAMP-dependent protein kinase A activity (*kin*$^-$ cells) (24). Forskolin, which bypasses the receptor-coupling defect in the *unc* mutant, restores ethanol inhibition of uptake in *unc* cells, but not in *kin*$^-$ cells. These data indicate that ethanol inhibition of NBMPR-sensitive nucleoside transporters might be a result of phosphorylation of the transporter or an associated regulatory component. Recently, an eight-amino acid sequence has been described for the γ-Aminobutyric acid type A receptor that confers ethanol sensitivity (41); phosphorylation of a serine in this sequence appears to regulate the ethanol sensitivity (42). When cDNA and antibody probes for facilitative nucleoside transporters become available, it will be possible to identify the domain responsible for ethanol inhibition.

It is possible that there are subclasses of NBMPR-sensitive transporters, only some of which are sensitive to ethanol. Recently, Barros *et al.* (43) have detected at least two isoforms of NBMPR-sensitive transporters in human placenta. Molecular probes will enable us to determine whether all or only specific isoforms of NBMPR-sensitive nucleoside transporters are inhibited by ethanol.

In model cell systems, ethanol inhibition of adenosine uptake initiates a cascade of events leading to both heterologous desensitization of receptors coupled to adenylyl cyclase via G_S and to reduced cAMP levels (18). We find that lymphocytes from actively drinking alcoholics also have reduced receptor-stimulated cAMP levels when compared with lymphocytes from control subjects (22). Thus, an early effect of ethanol on human lymphocytes could be inhibition of adenosine uptake via NBMPR-sensitive facilitative nucleoside transporters, which would lead to heterologous desensitization of cAMP signal transduction in these cells. Identification of NBMPR-sensitive nucleoside transporters as an initial target of ethanol may enable the design of specific agents to prevent adenosine-mediated responses to ethanol.

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