0026-895X/94/061281-06$03.00/0

MOLECULAR PHARMACOLOGY, 45:1281-1286, 1994.

Selective Inhibition by Ethanol of the Type 1 Facilitative Glucose Transporter (GLUT1)

SHARON WALD KRAUSS,[1] IVAN DIAMOND, and ADRIENNE S. GORDON

Ernest Gallo Clinic and Research Center (S.W.K., I.D., A.S.G.), Departments of Neurology (S.W.K., I.D., A.S.G.), Pediatrics (I.D.), and Pharmacology (I.D., A.S.G.), and the Neuroscience Program (I.D., A.S.G.), University of California, San Francisco, California 94143

Received October 5, 1993; Accepted March 18, 1994

SUMMARY

Ethanol appears to modulate the function of selective mammalian receptors and transporters by interacting with highly specific membrane protein sites. Of the multiple types of nucleoside transporters known to be present in mammalian cells, we observed that ethanol inhibits only one class of facilitative nucleoside transporters, that inhibited by nitrobenzylmercaptopurine riboside. Because there are biochemical similarities between facilitative glucose transporters and nitrobenzylmercaptopurine riboside-sensitive nucleoside transporters, we tested whether ethanol might selectively inhibit a unique class of facilitative glucose transporters. We report here that ethanol inhibits hexose uptake in human lymphocytes and several cell lines expressing the ubiquitous facilitative type 1 glucose transporter (GLUT1). Ethanol inhibition of hexose uptake by GLUT1 is independent of ethanol inhibition of facilitative nucleoside transport. We also determined the ethanol sensitivity of various cloned human facilitative glucose transporters expressed in Chinese hamster ovary cells and we found that ethanol inhibits hexose uptake by GLUT1 but not uptake by GLUT3 or GLUT4 transporters. Our results suggest that a protein motif or motifs present in the GLUT1 amino acid sequence but absent in GLUT3 or GLUT4 proteins may confer ethanol sensitivity.

Ethanol exerts some of its biologic effects by modulating the function of specific membrane proteins. For example, ethanol activates nicotinic acetylcholine, γ-aminobutyric acid type A, and serotonin receptors (1–4) but inhibits *N*-methyl-D-aspartate receptors (5) and a voltage-sensitive K^+ channel (6). Our laboratory reported that ethanol inhibits adenosine uptake via nucleoside transporters (7) and demonstrated that ethanol inhibition is specific for one type of nucleoside transporter (8). We found that facilitative uptake of nucleosides is significantly inhibited by ethanol, whereas transport of nucleosides by two types of concentrative transporters (9) is not affected (8). Furthermore, not all types of facilitative transporters are inhibited; ethanol specifically inhibits uptake by those facilitative nucleoside transporters sensitive to NBMPR (8).

Glucose transporters resemble nucleoside transporters in many respects. Both can be divided into sodium-dependent, concentrative types and sodium-independent, facilitative types. Concentrative glucose transporters appear to be related to concentrative nucleoside transporters, having 61% identity and 80% similarity in amino acid sequences (10). In addition, facilitative glucose transport proteins behave almost identically to facilitative nucleoside transport proteins during many different purification procedures (all are band 4.5 polypeptides) (11–13). As in the case of facilitative nucleoside transporters, there are multiple facilitative glucose transporters; five cDNAs for facilitative glucose transporters (GLUT1–5) have been cloned to date. These have partial homology to each other and have characteristic tissue distributions (for reviews, see Refs. 14 and 15). Determination of homologies between facilitative nucleoside and facilitative glucose transporters awaits cloning and sequencing of genes for facilitative nucleoside transport proteins.

Because we observed a unique pattern of ethanol inhibition among the members of the facilitative nucleoside transport protein family, we predicted that specific facilitative glucose transporters might also be sensitive to ethanol. We report here that ethanol inhibits hexose uptake by GLUT1 transporters but not uptake by GLUT3 or GLUT4 transporters. Our data, together with sequence information for the glucose transporters, should allow us to define the molecular basis for specific interactions of ethanol with GLUT1 transporters.

Experimental Procedures

Materials. [^{3}H]2DG, [^{3}H]MG, and [^{3}H]adenosine were purchased from Amersham. NBMPR was from Calbiochem. Mineral oil was obtained from Fisher Scientific and silicone oil from Aldrich. All other

This work was supported by grants from the National Institute on Alcohol Abuse and Alcoholism.
[1] Current address: Department of Cell and Molecular Biology, Mail Stop 74-157, University of California, Lawrence Berkeley Laboratory, Berkeley, CA 94720.

ABBREVIATIONS: NBMPR, nitrobenzylmercaptopurine riboside; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); 2DG, 2-deoxy-D-glucose; MG, 3-*O*-methyl-D-glucose; CHO, Chinese hamster ovary; GLUT1–5, facilitative glucose transporter types 1–5.

reagent-grade chemicals were purchased from Sigma or Boehringer-Mannheim. ExCell was obtained from J.R. Scientific and NuSerum from Collaborative Research. Other cell culture media were the products of GIBCO.

Cell culture. Human lymphocytes isolated from nonalcoholic control subjects were cultured in defined medium containing growth factors to stimulate proliferation, as described previously (16). S49 wild-type cells and Hep G2 cells (obtained from the Cell Culture Facility at the University of California, San Francisco), as well as the S49 mutants 160-D4 and PH-S-M (the kind gift of Dr. Buddy Ullman, University of Oregon), were cultured in Dulbecco's modified Eagle medium with 10% NuSerum or 10% ExCell. NG108-15 cells were grown in defined medium as described (17). The CHO line DG-44 (parent) and CHO cells transfected with cloned human glucose transporters, i.e., G3 (GLUT3) and G4–7 (GLUT4), were very generously provided by Dr. Charles F. Burant, Howard Hughes Institute, University of Chicago Medical Center. DG-44 cells were grown in Dulbecco's modified Eagle medium with 10% heat-inactivated fetal calf serum, and the transfected lines were cultured in riboside-free modified Eagle medium with 10% dialyzed fetal calf serum and 1 μM methotrexate. CHO lines K1 (parent) and GT3 (transfected with human GLUT1) were the kind gifts of Dr. Michael P. Czech, University of Massachusetts Medical Center (18), and were cultured in Ham's F12 medium containing 10% fetal calf serum.

Uptake measurements. Cells grown in suspension were centrifuged, and the pellet was resuspended at 2–5 × 10^7 cells/ml in phosphate-buffered saline containing 25 mM HEPES, pH 7.5, and was preincubated for 4 min at room temperature with either buffer alone, alcohols, cytochalasin B, or other inhibitors. Uptake was initiated by the addition of 100 μl of cells to 100 μl of assay medium, with incubation for the indicated times. Reaction mixtures contained either 60 μM [^{3}H]2DG (2 μCi/ml), 30 μM [^{3}H]MG (2 μCi/ml), or 0.3 μM [^{3}H]adenosine (1 μCi/ml). Specificity for uptake of D- and not L-glucose was also confirmed. Ethanol was removed from [^{3}H]2DG before use by evaporation using nitrogen. Uptake reactions were stopped by pelleting cells through inert oil and were then processed as described previously (7). Experiments to determine sodium-dependent uptake (Fig. 1A) were carried out in buffers containing 5 mM Tris, pH 7.5, 1 mM $MgCl_2$, and either 145 mM NaCl (sodium-containing) or 145 mM choline chloride (sodium-free).

For attached cells, culture media were aspirated from the wells and the cells were preincubated as described above. After removal of radioactive uptake media, cells were immediately rinsed with 3 ml of ice-cold phosphate-buffered saline (NG108-15) or rinsed three times with 1 ml of cold phosphate-buffered saline (Hep G2 and CHO) and dissolved in 1 ml of 2 N NaOH. The plates were incubated overnight at room temperature. The cell lysate was titurated, and aliquots were placed in scintillation vials, neutralized with HCl, mixed with scintillation fluid, and counted.

Results

Ethanol inhibits hexose uptake in cells expressing GLUT1. The GLUT1 glucose transporter is the most ubiquitous member of the facilitative glucose transporter family. It was the first to be cloned (19) and is expressed in Hep G2 cells, erythrocytes, lymphocytes, brain, placenta, and most cultured cell lines. We measured uptake of the glucose analog [^{3}H]2DG in cultured human lymphocytes. Uptake of [^{3}H]2DG was linear for at least 30 sec and facilitative, because it was both inhibited by cytochalasin B (see legend to Fig. 1) and independent of sodium (Fig. 1A).

The effect of 25–200 mM ethanol on linear [^{3}H]2DG uptake in human lymphocytes was also examined (Fig. 1B). Inhibition of [^{3}H]2DG uptake was significant with 25 mM ethanol, a clinically relevant concentration, and increased with increasing

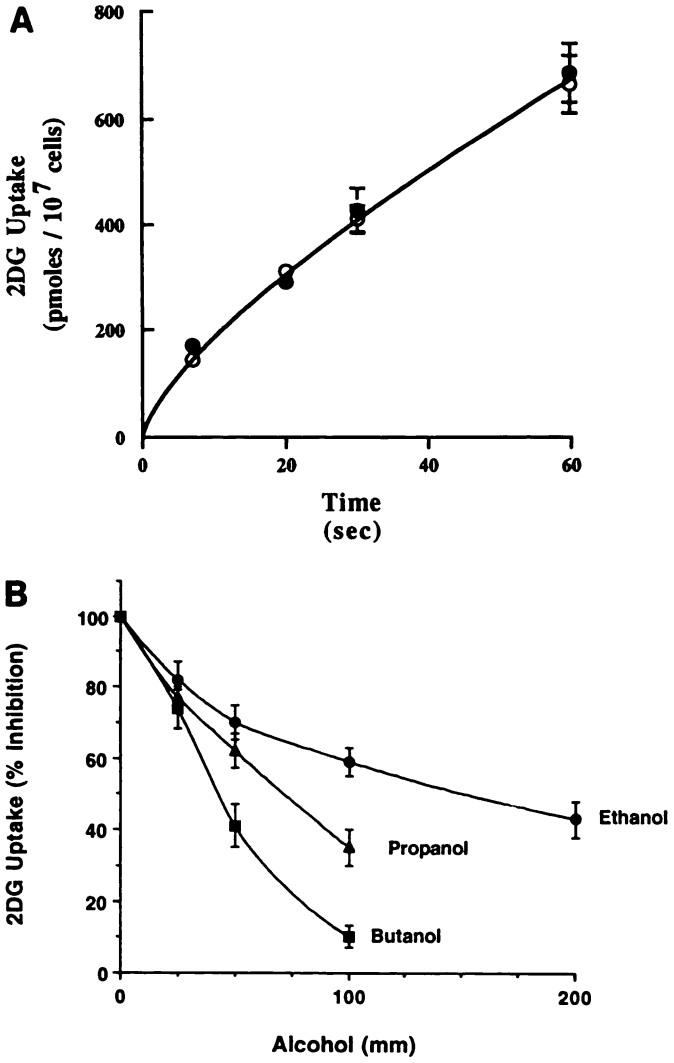

Fig. 1. Uptake of 2DG by cultured human lymphocytes. A, Time course of [^{3}H]2DG uptake in cultured human lymphocytes. Assays were carried out as described in Experimental Procedures. Values reported are the mean ± standard deviation of triplicate determinations of a representative experiment (of three experiments), corrected for nonspecific uptake in the presence of 20 μM cytochalasin B. Values for nonspecific uptake were as follows: 7 sec, 91 pmol/10^7 cells; 20 sec, 129 pmol/10^7 cells; 30 sec, 146 pmol/10^7 cells; 60 sec, 170 pmol/10^7 cells. ○, sodium-containing medium; ●, sodium-free medium. B, Effect of ethanol, *n*-propanol, and *n*-butanol on uptake of [^{3}H]2DG in cultured human lymphocytes. Uptake of [^{3}H]2DG was measured at 12 sec after a 4-min preincubation with buffer or alcohols at the concentrations indicated. Values reported are from a representative experiment (of two experiments) and are relative to the buffer control (297 pmol/10^7 cells), from which nonspecific uptake in the presence of cytochalasin B (104 pmol/10^7 cells) was subtracted. Measurements were performed in triplicate and the errors represent standard deviations.

concentrations up to 200 mM. We also measured [^{3}H]2DG uptake in the presence of varying concentrations of alcohols with increasing chain lengths. Iso-osmotic concentrations of *n*-propanol and *n*-butanol were more potent inhibitors than ethanol (Fig. 1B), suggesting that inhibition by short-chain alcohols was not due to an osmotic effect but was more directly related to chain length.

We further confirmed ethanol inhibition of hexose uptake in human lymphocytes by using a nonmetabolizable glucose analog, MG. In these experiments, 200 mM ethanol inhibited

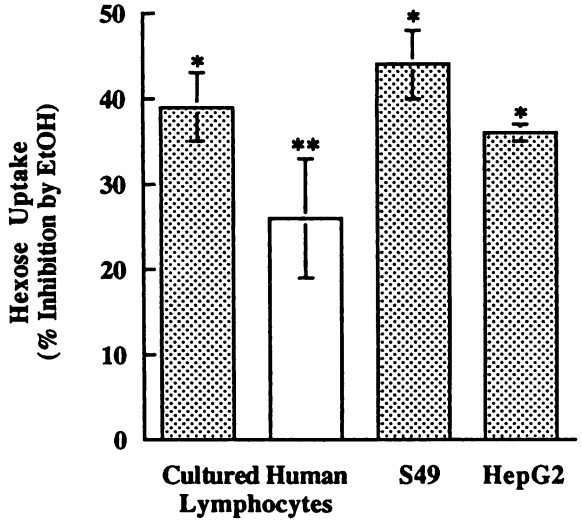

Fig. 2. Uptake of hexose by cells expressing GLUT1. Ethanol (*EtoH*) inhibition of [^{3}H]hexose uptake was measured after a 4-min preincubation with 200 mM ethanol. All assays were performed in triplicate in the linear range for uptake ([^{3}H]2DG, 10 sec for cultured human lymphocytes, 45 sec for S49 cells, and 12 sec for Hep G2 cells; [^{3}H]MG, 5 sec for cultured human lymphocytes). The results were normalized to uptake in the absence of ethanol, after correction for nonspecific uptake in the presence of 20 μM cytochalasin B. The values represent the mean ± standard error of 11 experiments with [^{3}H]2DG (seven different lymphocyte donors) and six experiments with MG (four different lymphocyte donors); for lymphocytes, nine experiments for S49 cells, and four experiments for Hep G2 cells. ▨, DG uptake; □, MG uptake. In all cell types, inhibition of hexose uptake was significant (*, $p < 0.0001$; **, $p < 0.0007$; Student's *t* test).

uptake of [^{3}H]MG by 26 ± 7% at 5 sec, compared with 39 ± 4% inhibition of [^{3}H]2DG uptake (Fig. 2). Because MG is not phosphorylated after entry into the cell and ethanol inhibition is similar to inhibition of [^{3}H]2DG uptake ($p > 0.05$, Student's *t* test), these results suggest that inhibition of hexose uptake by ethanol is due not to an effect of ethanol on glucose metabolism but to a direct effect of ethanol on GLUT1.

Ethanol also inhibited [^{3}H]2DG and [^{3}H]MG uptake in two other types of GLUT1-expressing cells, murine lymphoma S49 cells[2] and human hepatoma Hep G2 cells (Fig. 2 and data not shown). We used [^{3}H]2DG in most of the following experiments, however, because of its longer linear uptake range and more favorable ratio of uptake to background signal, compared with [^{3}H]MG.

Ethanol inhibition of GLUT1 transport is independent of ethanol inhibition of nucleoside uptake. Ethanol inhibits the uptake of adenosine and other nucleosides in many types of cells (7, 8, 20).[3] Although there are diverse classes of nucleoside transporters, we have reported that ethanol specifically inhibits only one class of nucleoside transporters, the NBMPR-sensitive facilitative type (8). Nucleosides have a five-membered carbon ribose ring, resembling the six-membered ring of glucose. In human erythrocytes, nucleosides can interact with the glucose transporter (21). Therefore, it is possible that nucleoside transporters might transport the smaller glucose molecule across the plasma membrane. If this occurs, then ethanol inhibition of glucose uptake might reflect inhibition of uptake via nucleoside transporters rather than glucose transporters.

We eliminated this possibility by three different types of experiments. First, inhibition of 2DG uptake by ethanol was determined in the presence of NBMPR at a concentration that inhibits facilitative NBMPR-sensitive nucleoside uptake in human lymphocytes, S49 cells, and Hep G2 cells. Under conditions where nucleoside transport was nearly completely inhibited by NBMPR (10 nM), [^{3}H]2DG uptake in all three cell lines was similar to that in untreated cells and was inhibited by ethanol (Table 1). Second, uptake of [^{3}H]2DG was measured in S49 cells and human lymphocytes in the presence of excess nonradioactive nucleoside (2 mM uridine), which would block any [^{3}H]2DG uptake by nucleoside transporters. Again, [^{3}H]2DG uptake was similar to that in control cells and was inhibited by ethanol (Table 1). Third, we measured [^{3}H]2DG uptake in two S49 mutant cell lines that are unable to transport nucleosides (PH-S-M and 160-D4). These mutant cells transported [^{3}H]2DG at levels similar to those of their wild-type parent (Table 1, legend) and, as in the parental cells, uptake of [^{3}H]2DG was inhibited by ethanol (Table 1). Thus, it appears that ethanol inhibition of [^{3}H]2DG uptake does not result from inhibition of [^{3}H]2DG influx via the nucleoside transporter but is an independent inhibitory effect of ethanol on GLUT1 transporters.

Ethanol does not inhibit uptake of hexose by GLUT3 or GLUT4. Based on our previous observations that only NBMPR-sensitive nucleoside transporters are inhibited by ethanol, we predicted that there might also be specificity of

TABLE 1

Ethanol inhibition of 2DG uptake is independent of nucleoside transport

[^{3}H]2DG uptake was determined in the absence or presence of 200 mM ethanol (EtOH), 10 nM NBMPR, or 2 mM uridine, as indicated. Experiments measuring nucleoside uptake were performed in parallel, as controls. Uptake was measured at 12 sec in cultured human lymphocytes or Hep G2 cells and at 45 sec in S49 cells. The results of two to eight determinations in triplicate were normalized to uptake in the absence of ethanol, after correction for nonspecific uptake with 20 μM cytochalasin B [100% values (mean ± standard error) were 189 ± 9 pmol/10^7 cells for lymphocytes, 244 ± 9 pmol/mg of protein for Hep G2 cells, 135 ± 8 pmol/10^7 cells for wild-type S49 cells, 146 ± 7 pmol/10^7 cells for PH-S-M cells, and 108 ± 6 pmol/10^7 cells for 160-D4 cells]. Uptake of the nonmetabolizable nucleoside analog formycin was <2.6 pmol/10^7 cells for the nucleoside transport-deficient S49 cell lines.

Cells	Conditions	Inhibition of [^{3}H]2DG uptake
		%
Cultured human lymphocytes	EtOH	32 ± 4.2
	NBMPR	6 ± 2
	NBMPR + EtOH	32 ± 1.5
	Uridine	2 ± 1
	Uridine + EtOH	27 ± 1.8
Hep G2 cells	EtOH	38 ± 1.8
	NBMPR	8 ± 1.8
	NBMPR + EtOH	42 ± 1.8
S49 wild-type cells	EtOH	38 ± 3.5
	NBMPR	14 ± 1.8
	NBMPR + EtOH	45 ± 3
	Uridine	3 ± 3
	Uridine + EtOH	38 ± 4.5
S49 nucleoside transport mutants		
160-D4 cells	EtOH	32 ± 1.8
PH-S-M cells	EtOH	30 ± 1.2

[2] Although 2DG uptake in S49 cells was reported to be ethanol insensitive (7), this result was in error due to ethanol being present in the radioactive preparation of [^{3}H]2DG. When ethanol is removed from the radioactive sample and uptake is performed in glucose-free medium, ethanol inhibits 2DG uptake in these cells.

[3] M. K. Sapru, I. Diamond, and A. S. Gordon. Adenosine receptors mediate cellular adaptation to ethanol in neuroblastoma × glioma hybrid cells. Submitted for publication.

ethanol inhibition among members of the facilitative glucose transporter family. NG108-15 is a neuroblastoma × glioma cell line that we have used as a model system to study acute and chronic effects of ethanol on cAMP signal transduction and nucleoside transport (17).[3] These cells have predominantly GLUT3 transporters, with ~25% GLUT1 expression (22). We first established optimal conditions for uptake of [^{3}H]2DG in NG108-15 cells (Fig. 3) and then determined whether this uptake was inhibited by ethanol. We found that concentrations as high as 300 mM ethanol did not inhibit uptake of [^{3}H]2DG at 40 sec, although nucleoside uptake was inhibited 34 ± 3% (four experiments) by 200 mM ethanol in these cells. [^{3}H]2DG uptake at 40 sec was 457 ± 14, 453 ± 27, 474 ± 24, 460 ± 15, and 459 ± 32 pmol/mg with 0, 50, 100, 200, and 300 mM ethanol, respectively. Because endogenous GLUT1 contributes only 25% to this uptake, it would be difficult to observe 25–35% inhibition of this activity over that of the predominant GLUT3 transporter. These data suggest that hexose uptake via GLUT3 is resistant to inhibition by ethanol.

To test the relative ethanol sensitivity of various facilitative glucose transporters in the same cell type, we measured the effect of ethanol on [^{3}H]2DG uptake in CHO cells transfected with cloned human GLUT1, GLUT3, and GLUT4 transporters (Fig. 4A). In all three transfected cell lines, [^{3}H]2DG uptake increased up to at least 60 sec. Uptake by GLUT1-overexpressing cells was 10 times that of their parent CHO cell line, whereas that of the GLUT3- and GLUT4-overexpressing cell lines was 6-fold and 3-fold increased, respectively, compared with their corresponding parent cell lines. 2DG uptake by endogenous hamster GLUT1 in CHO cells was sensitive to ethanol (Fig. 4B, *DG44* and *K1 parents*), consistent with our results in other GLUT1-containing cells (Fig. 2). Uptake of [^{3}H]2DG by CHO cells that overexpress human GLUT1 was also inhibited by ethanol (Fig. 4B). However, uptake of [^{3}H]2DG by CHO cells that overexpress GLUT3 or GLUT4 was not inhibited by ethanol under the same conditions (Fig. 4B). To confirm that ethanol inhibition of hexose uptake occurs at other hexose concentrations, we measured ethanol inhibition of [^{3}H]2DG at varying concentrations of 2DG. Ethanol inhibited uptake of 0.5, 1, and 5 mM [^{3}H]2DG in the GLUT1-overexpressing cells by 38 ± 7, 43 ± 8, and 32 ± 5% (mean ± standard deviation), respectively. No significant inhibition of the GLUT3- and GLUT4-expressing cell lines was detected at these concentrations of permeant (GLUT3, 6 ± 6, 9 ± 10, and 2 ± 7%; GLUT4, 1 ± 11, −4 ± 7, and 8 ± 5%, respectively; uptake at each permeant concentration for GLUT1, GLUT3, and GLUT4 was measured in triplicate, in one experiment). As in the case of 2DG uptake in NG108-15 cells, uptake by endogenous GLUT1 contributed a relatively small fraction of

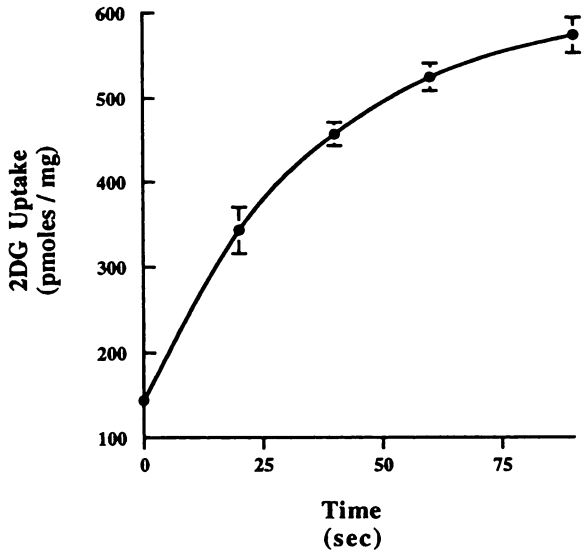

Fig. 3. Time course for uptake of [^{3}H]2DG in NG108-15 cells. Results are from a representative experiment (of three experiments) measuring [^{3}H]2DG uptake in triplicate (mean ± standard deviation) at the times indicated.

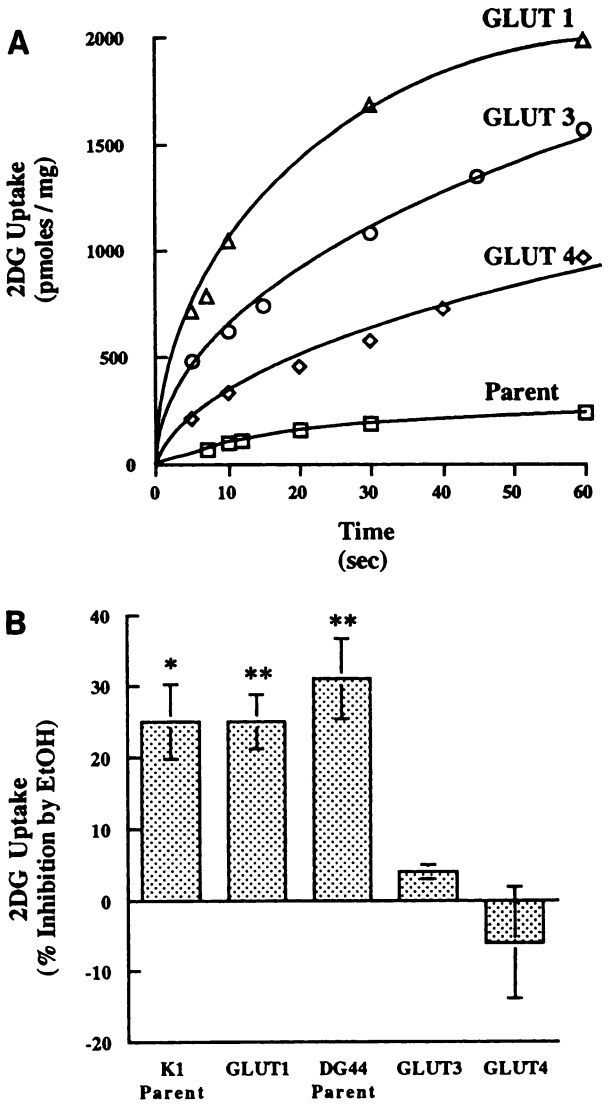

Fig. 4. Uptake of [^{3}H]2DG by CHO cells. A, Time course for uptake of [^{3}H]2DG in parental CHO cells and CHO cells overexpressing GLUT1, GLUT3, or GLUT4. Assays were done in triplicate and each time point is the average of two to seven determinations. For clarity, standard errors (3–10%) were omitted. Values were corrected for nonspecific uptake using cytochalasin B (DG44 and K1 parent cell lines, 26 pmol/mg of protein; GLUT1, 112 pmol/mg of protein; GLUT3, 84 pmol/mg of protein; GLUT4, 64 pmol/mg of protein). B, Effect of ethanol (*EtoH*) on [^{3}H]2DG uptake. Cells were preincubated for 4 min in the presence or absence of 200 mM ethanol and uptake in the linear range of [^{3}H]2DG was determined after 10 sec (parent) or 5 sec (overexpressing CHO lines). The values represent the mean ± standard error corrected for nonspecific uptake (DG-44, four experiments; K1, three experiments; GLUT1, eight experiments; GLUT3 and GLUT4, five experiments). *, $p < 0.0014$; **, $p < 0.0003$; (Student's *t* test).

the total uptake of [^{3}H]2DG in the transfected cells; ethanol inhibition of endogenous GLUT1 uptake was not detected in GLUT3- and GLUT4-overexpressing cells. Ethanol inhibited NBMPR-sensitive facilitative nucleoside uptake in all of the CHO cell lines.[4] Therefore, as predicted, ethanol has selective effects on members of the facilitative glucose transporter family; hexose uptake by GLUT1 is inhibited by ethanol but ethanol does not inhibit uptake by GLUT3 or GLUT4.

Discussion

Although many effects of ethanol have been attributed to generalized perturbation of cell membrane lipids, recent evidence indicates that ethanol can interact specifically with sites on selective proteins to alter their function. For example, ethanol binds to a hydrophobic site on nicotinic acetylcholine receptors that causes activation (1). In some cases, ethanol sensitivity results from a more complex set of events. Ethanol sensitivity of γ-aminobutyric acid type A receptors requires the presence of an alternatively spliced γ subunit containing an extra eight amino acids (3) and appears to depend on phosphorylation of a serine within that sequence (23). Our laboratory found that only one type of facilitative nucleoside transporter is inhibited by ethanol (8) and that ethanol sensitivity of nucleoside uptake by this transporter is dependent on protein kinase A activity in S49 cells (23a). Thus, some biologic effects of ethanol may result from interactions at a specific protein site that are regulated by phosphorylation.

Glucose is transported into mammalian cells by proteins biochemically very similar to facilitative nucleoside transport proteins. At least five types of facilitative glucose transporters have been identified. Previous reports described ethanol inhibition of hexose uptake in cultured astrocytes (24) and in adipocyte membranes (25). However, these studies did not address which types of glucose transporters were inhibited. To determine the effects of ethanol on individual transporters, we have used stable transfectants overexpressing human GLUT isoforms. Here we report our studies that document the specificity of ethanol inhibition for one of the members of the facilitative glucose transporter family. We find that ethanol inhibits hexose uptake by GLUT1 transporters but not uptake by GLUT3 or GLUT4.

Nucleoside transporter proteins transport molecules containing the sugar ribose. To exclude the possibility that ethanol inhibition of [^{3}H]2DG uptake reflects uptake via ethanol-sensitive nucleoside transporters, we used chemical inhibitors of nucleoside transport or mutant cells lacking nucleoside transport activity. Our results directly demonstrate that in cultured human and murine cells nucleoside transporters do not accept [^{3}H]2DG as a permeant, consistent with a previous observation that excess D-glucose did not significantly inhibit uridine uptake into human erythrocytes (21). Therefore, we conclude from our data (Table 1) that ethanol inhibits hexose uptake by GLUT1, independently of nucleoside transport.

We also determined that inhibition of [^{3}H]2DG uptake is specific for GLUT1 by taking advantage of CHO cell lines that overexpress individually transfected human glucose transporter genes. 2DG uptake in these cells was 3–10-fold higher than uptake by endogenous CHO GLUT1 present in wild-type parent cells (Fig. 4A), confirming functional overexpression of the transfected human glucose transporters. Uptake of [^{3}H]2DG in both untransfected CHO cells (DG44 and K1) and human GLUT1-transfected CHO cells was inhibited by ethanol, demonstrating that overexpression *per se* does not alter ethanol sensitivity of GLUT1. In contrast to cells expressing GLUT1, [^{3}H]2DG uptake in CHO cells overexpressing human GLUT3 or GLUT4 facilitative transporters was not inhibited by 200 mM ethanol (Fig. 4B) nor was uptake via GLUT3 transporters in NG108-15 cells perturbed by even 300 mM ethanol. Furthermore, ethanol sensitivity of hexose uptake is not dependent on the growth medium, because GLUT1-expressing cells were grown in a variety of defined and serum-containing media (Figs. 1, 2, and 4). Our results also rule out ethanol effects on hexokinase activity, the initial metabolic step after uptake of glucose, because (a) ethanol inhibited MG as well as [^{3}H]2DG uptake in GLUT1-expressing cells (Fig. 2 and data not shown) and MG is not phosphorylated by hexokinase, and (b) [^{3}H]2DG uptake was unaffected by ethanol in NG108-15 cells expressing predominantly GLUT3, as well as CHO cells overexpressing GLUT3 and GLUT4. All of these cell lines have hexokinase activity. Because ethanol selectively inhibits only one type of glucose transporter when the transporter is expressed in the same cellular background as two other types, our data suggest that ethanol inhibition of hexose uptake is determined by the amino acid sequence of the GLUT1 transporter protein.

The cDNAs encoding the five structurally related sodium-independent facilitative glucose transporter proteins have been sequenced; each is the product of a separate gene (reviewed in Refs. 14, 15, and 26). The transporter isoforms have distinct tissue specificities, although they may be expressed separately, together, or with sodium-dependent concentrative glucose transporters. The facilitative transporter proteins are all ~500 amino acids long and have 12 membrane-spanning segments with intracellular amino and carboxyl termini, a large extracellular loop containing a consensus sequence for glycosylation, and a hydrophilic intracellular loop (15, 19). Despite overall structural and functional similarities, amino acid sequences of GLUT transporters vary considerably. There is 39–65% sequence identity and 50–76% sequence similarity between the GLUT1–5 isoforms; 26% of the residues are invariant, with another 13% representing conservative amino acid replacements. The most divergent regions are the amino and carboxyl termini and the large extracellular domain between the first two transmembrane segments.

Human GLUT3 and GLUT4 are ~65% homologous to human GLUT1 and 58% homologous to each other. Interestingly, the amino acid sequences of GLUT1 and GLUT4 are each very highly conserved (>95%) in human, rat, rabbit, mouse, and pig (19, 27–30), implying that all domains of both of these proteins are functionally important.

The facilitative glucose transporters are differentially regulated by a variety of agents such as insulin or growth hormone. However, GLUT1 appears to be responsible for constitutive glucose uptake in virtually all tissues, because low levels of GLUT1 protein or mRNA were detected in all tissues tested. In cultured cells and many tumors, GLUT1 is the predominant facilitative glucose transporter (14, 15). Reducing uptake of glucose into cells by ethanol exposure could compromise vital metabolic processes. In addition, because glucose metabolism regulates intracellular Ca^{2+}, cAMP, and diacylglycerol concen-

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

trations (31), inhibition of glucose uptake by ethanol might alter protein kinase A and protein kinase C activities as well.

To understand the molecular basis of ethanol inhibition of GLUT1 transporters, chimeras of ethanol-sensitive and ethanol-insensitive facilitative glucose transporters could be used. This approach is currently being used successfully to deduce the GLUT4 domain(s) critical for insulin regulation and the regions of GLUT1 involved in oligomer formation (28, 32, 33). If a GLUT1 domain that confers ethanol sensitivity when spliced to GLUT3 and GLUT4 sequences could be identified, then it would be possible to map crucial amino acids by systematic site-specific mutagenesis. Definition of an ethanol-sensitive domain could suggest a biochemical mechanism for ethanol inhibition of hexose transport by GLUT1. Furthermore, similar ethanol-specific protein motifs might also be present in the ethanol-sensitive facilitative nucleoside transporter and other ethanol-sensitive membrane proteins (1–8).

Acknowledgments

We are very grateful to Drs. Charles F. Burant (University of Chicago Medical Center) and Michael P. Czech (University of Massachusetts Medical Center) for CHO cell lines used in this study, Dr. Mohan Sapru (University of Michigan) for advice on experiments with NG108-15 cells, Ms. Janet Michelwait for assistance with cell culturing, and Dr. Carol Cass (University of Alberta, Canada) for helpful discussions.

References

1. Wood, S. C., S. A. Forman, and K. W. Miller. Short chain and long chain alkanols have different sites of action on nicotinic acetylcholine receptor channels from *Torpedo*. *Mol. Pharmacol.* **39**:332–338 (1991).
2. Mehta, A. K., and M. K. Ticku. Are $GABA_B$ receptors involved in the pharmacological effects of ethanol? *Eur. J. Pharmacol.* **182**:473–480 (1990).
3. Wafford, K. A., D. M. Burnett, N. J. Leidenheimer, D. R. Burt, J. B. Wang, P. Kofuji, T. V. Dunwiddie, R. A. Harris, and J. M. Sikela. Ethanol sensitivity of the $GABA_A$ receptor expressed in *Xenopus* oocytes requires 8 amino acids contained in the γ2L subunit. *Neuron* **7**:27–33 (1991).
4. Lovinger, D. M., and G. White. Ethanol potentiation of 5-hydroxytryptamine$_3$ receptor-mediated ion current in neuroblastoma cells and isolated adult mammalian neurons. *Mol. Pharmacol.* **40**:263–270 (1991).
5. Lovinger, D. M., G. White, and F. F. Weight. NMDA receptor-mediated synaptic excitation selectively inhibited by ethanol in hippocampal slices from adult rat. *J. Neurosci.* **10**:1372–1379 (1990).
6. Covarrubias, M., and E. Rubin. Ethanol selectively blocks a noninactivating K^+ current expressed in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **90**:6957–6960 (1993).
7. Nagy, L. E., I. Diamond, D. J. Casso, C. Franklin, and A. S. Gordon. Ethanol increases extracellular adenosine by inhibiting adenosine uptake via the nucleoside transporter. *J. Biol. Chem.* **265**:1946–1951 (1990).
8. Krauss, S. W., R. B. Ghirnikar, I. Diamond, and A. S. Gordon. Inhibition of adenosine uptake by ethanol is specific for one class of nucleoside transporters. *Mol. Pharmacol.* **44**:1021–1026 (1993).
9. Vijayalakshmi, D., and J. A. Belt. Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. *J. Biol. Chem.* **263**:19419–19423 (1988).
10. Pajor, A. M., and E. M. Wright. Cloning and functional expression of a mammalian Na^+/nucleoside cotransporter. *J. Biol. Chem.* **267**:3557–3560 (1992).
11. Klip, A., and D. Walker. Chemical and genetic comparison of the glucose and nucleoside transporters. *Biochem. Cell Biol.* **64**:1170–1180 (1986).
12. Tse, C.-M., J. A. Belt, S. M. Jarvis, A. R. P. Paterson, J.-S. Wu, and J. D. Young. Reconstitution studies of the human erythrocyte nucleoside transporter. *J. Biol. Chem.* **260**:3506–3511 (1985).
13. Kwong, F. Y. P., A. Davies, C. M. Tse, J. D. Young, P. J. F. Henderson, and S. A. Baldwin. Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem. J.* **255**:243–249 (1988).
14. Pessin, J. E., and G. I. Bell. Mammalian facilitative glucose transporter family: structure and molecular regulation. *Annu. Rev. Physiol.* **54**:911–930 (1992).
15. Silverman, M. Structure and function of hexose transporters. *Annu. Rev. Biochem.* **60**:757–794 (1991).
16. Nagy, L. E., I. Diamond, and A. Gordon. Cultured lymphocytes from alcoholics have altered cAMP signal transduction. *Proc. Natl. Acad. Sci. USA* **85**:6973–6976 (1988).
17. Gordon, A. S., K. Collier, and I. Diamond. Ethanol regulation of adenosine receptor-dependent cAMP levels in a clonal neural cell line: an *in vitro* model of cellular tolerance to ethanol. *Proc. Natl. Acad. Sci. USA* **83**:2105–2108 (1986).
18. Harrison, S. A., J. M. Buxton, and M. P. Czech. Suppressed intrinsic catalytic activity of GLUT1 glucose transporters in insulin-sensitive 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA* **88**:7839–7843 (1991).
19. Mueckler, M., C. Caruso, S. A. Baldwin, M. Panico, I. Blench, H. R. Morris, W. J. Allard, G. E. Lienhard, and H. F. Lodish. Sequence and structure of a human glucose transporter. *Science (Washington D. C.)* **229**:941–945 (1985).
20. Nagy, L. E. Ethanol metabolism and inhibition of nucleoside uptake lead to increased extracellular adenosine in hepatocytes. *Am. J. Physiol.* **262**:C1175–C1180 (1992).
21. Jarvis, S. M. Inhibition by nucleosides of glucose-transport activity in human erythrocytes. *Biochem. J.* **249**:383–389 (1988).
22. Maher, R., S. Vannucci, J. Takeda, and I. A. Simpson. Expression of mouse-GLUT3 and human-GLUT3 glucose transporter proteins in brain. *Biochem. Biophys. Res. Commun.* **182**:703–711 (1992).
23. Wafford, K. A., and P. J. Whiting. Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the γ2 subunit. *FEBS Lett.* **313**:113–117 (1992).
23a. Nagy, L. E., I. Diamond, and A. S. Gordon. cAMP-dependent protein kinase regulates inhibition of adenosine transport by ethanol. *Mol. Pharmacol.* **40**:812–817 (1991).
24. Singh, S. P., A. K. Snyder, and S. Eman. Effects of ethanol on hexose uptake by cultured rat brain cells. *Alcohol. Clin. Exp. Res.* **14**:741–745 (1990).
25. Hutchinson, B. T., P. A. Hyslop, C. E. Kuhn, and R. D. Sauerheber. Sensitivity of adipocyte basal and insulin-stimulated hexose transport to the membrane lipid structure. *Biochem. Pharmacol.* **34**:1079–1086 (1985).
26. Burant, C. F., W. I. Sivitz, H. Fukumoto, T. Kayano, S. Nagamatsui, S. Seino, J. E. Pessin, and G. I. Bell. Mammalian glucose transporters: structure and molecular regulation. *Recent Prog. Horm. Res.* **47**:349–388 (1991).
27. Birnbaum, M. J. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* **57**:305–315 (1989).
28. Asano, T., K. Takata, H. Katagiri, K. Tsukuda, J.-L. Lin, H. Ishihara, K. Inukai, H. Hirano, Y. Yazaki, and Y. Oka. Domains responsible for the differential targeting of glucose transporter isoforms. *J. Biol. Chem.* **267**:19636–19641 (1992).
29. Kaestner, K. H., R. J. Christy, J. C. McLenithan, L. T. Braiterman, P. Cornelius, P. H. Pekala, and M. D. Lane. Sequence, tissue distribution and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA* **86**:3150–3154 (1989).
30. Weiler-Guttler, H., H. Zinke, B. Mockel, A. Frey, and H. G. Gassen. cDNA cloning and sequencing analysis of the glucose transporter from porcine blood-brain barrier. *Biol. Chem. Hoppe-Seyler* **370**:467–473 (1989).
31. Zawalich, W. S., and H. Rasmussen. Control of insulin secretion: a model involving Ca^{2+}, cAMP, and diacylglycerol. *Mol. Cell. Endocrinol.* **70**:119–137 (1990).
32. Pessino, A., D. N. Herbert, C. W. Woon, S. A. Harrison, B. M. Clancy, J. M. Buxton, A. Carruthers, and M. P. Czech. Evidence that functional erythrocyte-type glucose transporters are oligomers. *J. Biol. Chem.* **266**:20213–20217 (1991).
33. Burant, C. F., and G. I. Bell. Mammalian facilitative glucose transporters: evidence for similar substrate recognition sites in functionally active monomeric proteins. *Biochemistry* **31**:10414–10420 (1992).

Send reprint requests to: Sharon Wald Krauss, Department of Cell and Molecular Biology, Mail Stop 74-157, University of California, Lawrence Berkeley Laboratory, Berkeley, CA 94720.