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Effects of Barbiturates on Facilitative Glucose Transporters are Pharmacologically Specific and Isoform Selective

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Abstract. Barbiturates inhibit GLUT-1-mediated glucose transport across the blood-brain barrier, in cultured mammalian cells, and in human erythrocytes. Barbiturates also interact directly with GLUT-1. The hypotheses that this inhibition of glucose transport is (i) selective, preferring barbiturates over halogenated hydrocarbon inhalation anesthetics, and (ii) specific, favoring some GLUT-# isoforms over others were tested. Several oxy- and thio-barbiturates inhibited [^{3}H]-2-deoxyglucose uptake by GLUT-1 expressing murine fibroblasts with IC_{50}s of 0.2–2.9 mM. Inhibition of GLUT-1 by barbiturates correlates with their overall lipid solubility and pharmacology, and requires hydrophobic side chains on the core barbiturate structure. In contrast, several halogenated hydrocarbons and ethanol (all $\leq$10 mM) do not significantly inhibit glucose transport. The interaction of these three classes of anesthetics with purified GLUT-1 was evaluated by quenching of intrinsic protein fluorescence and displayed similar specificities and characteristics. The ability of barbiturates to inhibit other facilitative glucose transporters was determined in cell types expressing predominantly one isoform. Pentobarbital inhibits [^{3}H]-2-deoxyglucose and [^{14}C]-3-O-methyl-glucose uptake in cells expressing GLUT-1, GLUT-2, and GLUT-3 with IC_{50}s of ~1 mM. In contrast, GLUT-4 expressed in insulin-stimulated rat adipocytes was much less sensitive than the other isoforms to inhibition by pentobarbital (IC_{50} of >10 mM). Thus, barbiturates selectively inhibit glucose transport by some, but not all, facilitative glucose transporter isoforms.

Key words: Barbiturates — Glucose transport — Anesthetics — Pharmacologic specificity — Isoform selectivity — Binding

Introduction

Previous observations indicate that barbiturates, at anesthetic doses, uniformly suppress (~25%) Glc transfer across the blood-brain barrier (BBB) in rats (Gjedde & Rasmussen, 1980; Otsuka et al., 1991). The predominant facilitative Glc transporter of the BBB in vivo is GLUT-1 (*for reviews see:* Pardridge & Boado, 1993; Maher, Vannucci & Simpson, 1994; Vannucci, Maher & Simpson, 1997), and this transporter has been proposed to be a likely target of barbiturates at the BBB (Fenstermacher et al., 1995) and possibly at other cells that express GLUT-1. In support of this suggestion, Honkanen et al. (1995) demonstrated that barbiturates inhibit Glc transport in several cultured mammalian cells and human erythrocytes, and interact directly with purified GLUT-1. Inhibition of Glc transport by barbiturates was also shown to not correlate with a generalized alteration of the physical state (i.e., fluidity) of the lipid bilayer (Honkanen et al., 1995). Recently, in kinetic studies of human erythrocyte Glc transport (El-Barbary, Fenstermacher & Haspel, 1996), inhibition of GLUT-1 function by barbiturates has been suggested to be of the noncompetitive type and is likely to involve a preferential interaction of barbiturates with a form of the carrier that is not occupied by substrate. Taken in concert, these findings (Gjedde & Rasmussen, 1980; Otsuka et al., 1991; Honkanen et al., 1995; El-Barbary et al., 1996) suggest that

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Abbreviations: BBB, blood-brain barrier; FB, fluorescence buffer; FQ, fractional quenching; GLUT-#, type # of the mammalian facilitative Glc transporters

barbiturate inhibition of GLUT-1-mediated Glc transport may be pharmacologically and biochemically quite specific.

Several isoforms of the facilitative Glc transporters have been cloned and characterized (*for reviews, see* Baldwin, 1993; Gould & Holman, 1993; Carruthers, 1990). Although quite similar with respect to overall amino acid sequence and predicted transmembrane topology, these isoforms differ significantly with respect to their tissue and cellular distribution, hormonal and metabolic regulation, substrate and inhibitor specificity, substrate and inhibitor affinities, and kinetic properties. These differences suggest that some isoforms may exhibit differential sensitivity to inhibition by barbiturates.

Herein, the pharmacologic and isoform specificities of barbiturate inhibition of facilitative Glc transporters will be evaluated. First, experiments involving a comparison of the sensitivity of Glc transport in cultured murine fibroblasts, expressing predominantly GLUT-1, to a wide spectrum of barbiturates *vs.* a wide spectrum of halogenated hydrocarbon inhalation anesthetics (Fig. 1) are reported. Second, experiments involving a comparison of the quenching of the intrinsic fluorescence of purified GLUT-1 by these anesthetics are reported. Third, experiments involving a comparison of the sensitivity to barbiturates of Glc transport in several cultured and primary cell lines expressing different facilitative Glc transporter isoforms are reported.

Materials and Methods

Materials

With the exception of thiobutabarbital, methohexital, and the optical isomers of pentobarbital, barbiturates were obtained from Sigma Chemical (St. Louis, MO). Cytochalasin B, phloretin, 2-deoxy-D-Glc (dGlc), 3-O-methyl-D-Glc (MeGlc), D-Glc, porcine insulin, collagenase, fraction V bovine serum albumin, Trp, and most other reagents were also obtained from Sigma Chemical (St. Louis, MO). [^{3}H]-dGlc and [^{14}C]- MeGlc were purchased from Dupont NEN (Wilmington, DE). Thiobutabarbital was obtained from Byk Gulden (Konstanz, Germany). Methohexital was obtained from Eli Lily (Indianapolis, IN). Samples of the (+) and (−) optical isomers of pentobarbital were kindly provided by Dr. F. Ivy Carrol of Research Triangle Institute (Research Triangle Park, NC). Halothane was obtained from Halocarbon Laboratories (River Edge, NJ). Desflurane and isoflurane were obtained from Ohmeda Caribe (Guayama, PR). Enflurane was obtained from Anaquest (Madison, WI). Methoxyflurane was obtained from Pitman-Moore (Mandlein, IL). Ethanol was obtained from Aaper (Shelbyville, KY). Sealable plastic bags were obtained from Consolidated Plastics (Twinsburg, OH).

Tissue Culture and Cell Isolation

3T3-C2 murine fibroblasts were obtained and maintained as described by Honkanen et al. (1995). Primary neonatal rat cerebellar neurons and cortical astrocytes were isolated, maintained and cultured as described by Maher et al. (1991, 1996). Primary rat epididymal white adipocytes were isolated, maintained, and handled as described by Weber et al. (1988). 3T3-L1 murine preadipocytes were obtained, maintained, and differentiated into insulin responsive 3T3-L1 adipocytes as previously described for 3T3-442A cells (Silverman et al., 1989). G6 and G2 AtT20ins were kindly provided by Dr. C. Newgard (University of Texas, Dallas, TX) and maintained as described by Hughes et al. (1992). These "artificial β cells" originate from anterior pituitary tumors and express *high* levels of insulin, glucokinase, and GLUT-2 (Hughes et al., 1992). Human erythrocytes were isolated and handled as described by El-Barbary et al. (1996).

Glc Transport Assays

Uptake of [^{3}H]-dGlc by 3T3-C2 and 3T3-L1 cells was assayed as described previously (Honkanen et al., 1995; Silverman et al., 1989). Uptake of [^{3}H]-dGlc and [^{14}C]-MeGlc by rat neurons and AtT20ins cells was assayed as described by Maher et al. (1991, 1996). Uptake of [^{3}H]-dGlc and [^{14}C]-MeGlc by rat adipocytes was assayed as described by Weber et al. (1988). Unidirectional flux of Glc by human erythrocytes was assayed as described by El-Barbary et al. (1996).

Purification and Preparation of GLUT-1

GLUT-1 was purified from alkali-stripped human erythrocyte membranes and reconstituted into endogenous phospholipids as previously described by Baldwin and Lienhard (1989) with the modifications previously described by Honkanen et al. (1995). Purified and reconstituted GLUT-1 was dialyzed into fluorescence buffer (FB) consisting of 100 mM NaCl, 1 mM EDTA, and 75 mM HEPES, pH 7.4, and stored frozen at −70° in ~1 ml aliquots. Before use a fresh aliquot of GLUT-1 (~300 μg/ml) is rapidly thawed, probe sonicated at 50 W for 30 sec at 20°, to diminish light scattering, and stored at 4° for ≤24 hr.

Fluorescence Quenching Studies of Purified GLUT-1

Intrinsic fluorescence of purified GLUT-1 was measured essentially as described previously (Honkanen et al., 1995) with several modifications. Fluorescence measurements were carried out in the ratio mode with a SLM 8100 spectrofluorometer, equipped with a refrigerated photomultiplier tube, interfaced to a Dell Pentium microcomputer for data acquisition, storage, and manipulation with software from SLM (Rochester, NY). The cell compartment was generally thermostated at 20° and the sample contained in constantly stirred 1 ml quartz fluorescence cuvet. To reduce scattered light a 305 nm cutoff filter (WG-305; Schott Glass Technologies (Durwea, PA) was employed before the emission monochrometer. Excitation and emission slits were 1 and 4 mm, respectively. Single wavelength emission intensity determinations were taken for sequential titration studies to limit photobleaching. Six sequential integrations of signal intensity with an integration time of 1 sec were determined for each sample and the closest five averaged. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 280 and 380 nm, respectively. This choice of λ_{em}, although removed from the emission maximum of ~340 nm, makes the measurements more reproducible by further limiting light scattering artifacts. Typically, 1 ml of GLUT-1 (~25 μg/ml, ~400 nM) containing FB was placed in a cuvet and allowed to temperature equilibrate for 2 min before an initial fluorescence intensity was determined. The appropriate anesthetic or vehicle was added in small aliquots (1–25 μl) from concentrated stocks (~20 *X*) and fluorescence intensity determined after 2 min. The total volume change was always ≤5% of the initial volume. For each titration two individual samples were assayed. The initial and final (i.e., after the last addition of titrant) absorbance of the samples at λ_{ex} and λ_{em} were measured using a Beckman DU64 spectrophotometer (Fullerton, CA) and the values used for inner filter effect corrections.

For use in these intrinsic protein fluorescence studies, halothane and isoflurane were purified daily by passage over neutral alumina (Grade 1, Type WIN-3; Sigma Chemical, St. Louis, MO). This removes interference by the preservative thymol. Stock solutions (~200 mM) of halothane and isoflurane were then prepared in ethanol and solutions stored in gas tight septum flasks equipped with Teflon/Silicone seals (Pierce Chemical, Rockford, IL). To limit problems with uncontrollable changes in the concentrations of these volatile anesthetics, titrations with halothane and isoflurane were performed in custom-made (Hellma Cells, Forest Hills, NY) gas tight septum quartz fluorescence cuvets equipped with Teflon/Silicone seals. All solution transfers were made with gas tight micro syringes and the volume not occupied by aqueous sample was limited to ≤8%.

Fluorescence intensities and absorbance determinations were corrected for dilution. Intermediate absorbance values were calculated by

$$F_{cor} = F_{obs} * 10^{\frac{(A_{ex} + A_{em})}{2}} \quad (1)$$

$$FQ = 1 - \frac{F}{F_o} \quad (2)$$

$$FQ = \frac{FQ_{\max} * [L]}{K_d + [L]} \quad (3)$$

assuming the Beer-Lambert relationship (Poole & Bashford, 1987). Inner filter effect corrections of fluorescence intensities were made using the averaging approach outlined in Eq. 1 (Lackowicz, 1983) in which F_{cor} and F_{obs} are the corrected and observed intensities, respectively, and A_{em} and A_{ex} are the absorbencies at the emission and excitation wavelengths, respectively. Fractional quenching (FQ) was calculated from these corrected fluorescence intensities using Eq. 2 in which F_o and F are the initial and each subsequent F_{cor}, respectively. Means and SDs of FQ for three separate experiments each consisting of two titrated samples at each condition were than calculated. These values of FQ were assumed to be proportional to ligand/GLUT-1 complex and used to calculate apparent affinity (K_d) and maximal FQ (FQ_{max}) using a simple Langmuir isotherm (i.e., a rectangular/saturable hyperbolic function) as a binding model (Eq. 3, Hulme & Birdsall, 1993).

Results

GLUT-1 Mediated Glc Transport In 3T3-C2 Cells is Inhibited by a Wide Spectrum of Barbiturates but not by Halogenated Hydrocarbon Anesthetics

The concentration dependence for the inhibition of [^{3}H]-dGlc uptake by several oxy- (Fig. 2, *top panel*) and thio-barbiturates (Fig. 2, *bottom panel*) varies. All of the barbiturates examined inhibit Glc transport in 3T3-C2 cells at concentrations (IC_{50}s of 0.2–2.9 mM, Table 1) near to those used pharmacologically (Harvey, 1985; Firestone, Miller & Miller, 1986). Note that ~25% inhibition of Glc transport is observed at ~200 μM pentobarbital. This concentration is reasonably consistent with the content (~200 nmol/g tissue) of pentobarbital found in the brains of anesthetized rats (Richter & Waller, 1977). This concentration also correlates well with our previous in vivo observation (Otsuka et al., 1991) of ~25% suppression of Glc transfer across the BBB of pentobarbital anesthetized rats.

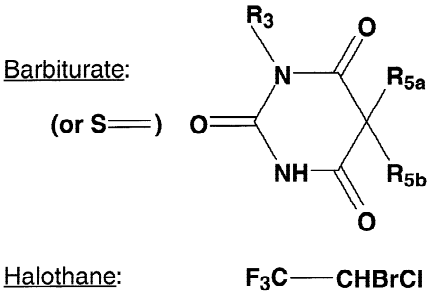


Fig. 1. General structural formula for barbiturates. The oxy- and thio-barbiturate core structures (i.e., 2,4,6-trioxohexahydropyrimidene and 2-thio,4,6-dioxohexahydropyrimidene, respectively) are depicted. Positions for potential alkyl or aryl groups at carbons 3 and 5 are also indicated (i.e., R_3, R_{5a}, and R_{5b}). This is a variation on the structure presented by Harvey (1985). The structure of halothane, a halogenated hydrocarbon anesthetic, is also depicted for comparison.

No distinct difference in the sensitivity of Glc transport to oxy- *vs.* thio-barbiturates is observed. Secobarbital (IC_{50} of 0.4 mM) and thiamylal (IC_{50} of 0.2 mM) were the most effective inhibitors of Glc transport. This order of potency for inhibition of Glc transport clearly, but loosely, adheres to the Meyer-Overton rule correlating overall lipid solubility with potency (Franks & Lieb, 1994). Plots of octanol/buffer or human erythrocyte membrane/buffer partition coefficients for barbiturates, where values are available (Firestone et al., 1986), *vs.* the IC_{50}s for inhibition of Glc transport by barbiturates are relatively linear with least squares linear regression analysis yielding correlation coefficients (r^2) of 0.818 and 0.894, respectively (*not shown*). These data are also generally consistent with the pharmacological potency of these barbiturates (Harvey, 1985; Firestone et al., 1986). No correlation of these IC_{50}s with barbiturates possessing either hypnotic, sedative, and/or convulsant activities (Harvey, 1985; Firestone et al., 1986) is evident. Other issues related to the overall size and flexibility, the so-called cutoff concept, of the alkyl/aryl side chains (i.e., positions R_3, R_{5a}, and R_{5b} of the core barbituric moiety; *see* Fig. 1) (Harvey, 1985; Firestone et al., 1986; Franks & Lieb, 1994) likely impact on these relative potencies in uncertain ways and therefore may influence the exact order of potency. The two core compounds, barbituric acid and thiobarbituric acid, do not significantly inhibit Glc transport at <10 mM. This suggests that inhibition of Glc transport by barbiturates absolutely depends, like many of the other effects of these agents (Harvey, 1985; Firestone et al., 1986; Franks & Lieb, 1994), upon the presence of substituents at R_3, R_{5a}, and/or R_{5b} on the parent structure. Furthermore, the IC_{50} for inhibition of Glc transport by pentobarbital (pK_a~7.8 at physiological ionic strengths) is increased by ~3-fold to 3 mM from 0.8 mM if the buffer pH is slightly raised to 8.2 from 7.4,

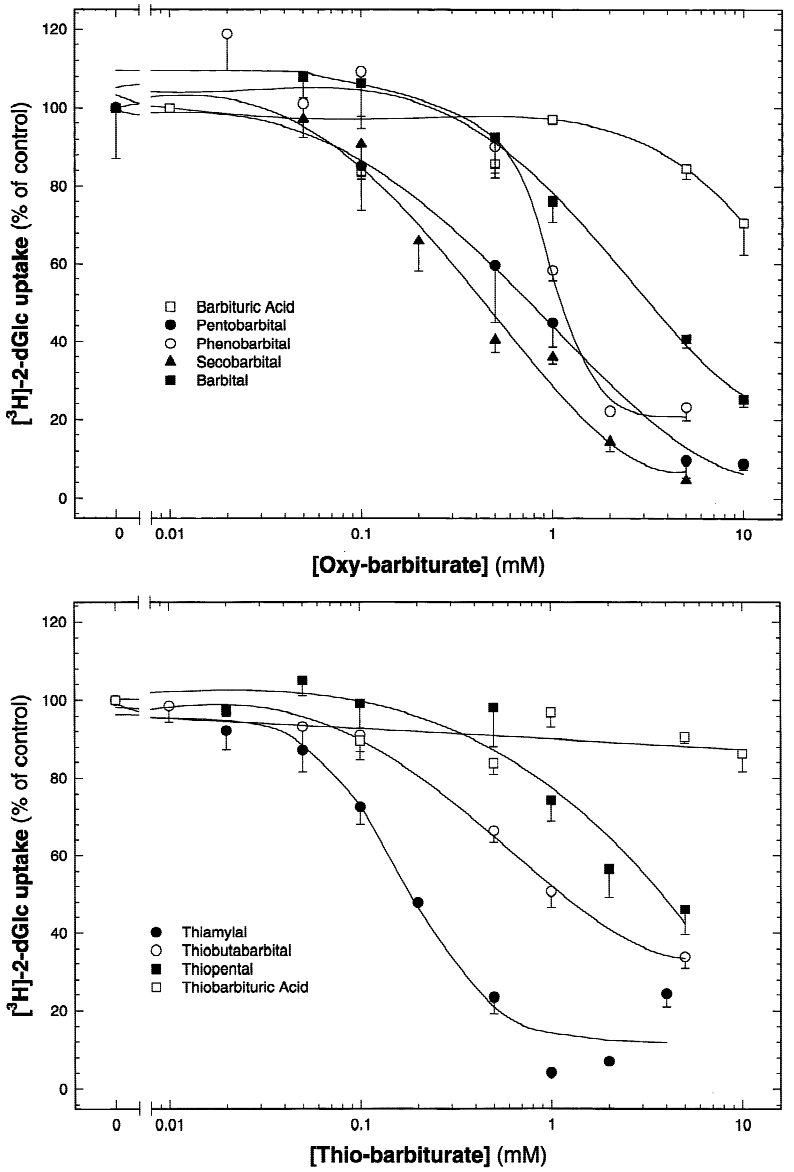


Fig. 2. Concentration dependence for the inhibition of Glc transport by oxy- and thio-barbiturates in 3T3-C2 murine fibroblasts. 3T3-C2 murine fibroblasts were grown to confluence in 35 mm six-well dishes and refed 12–24 hr before use. Cells were incubated at 23°C for 15 min in transport buffer (Honkanen et al., 1995) containing different concentrations of barbiturates. [^{3}H]-dGlc uptake was then assayed at 23° (Honkanen et al., 1995) in triplicate, for 15 min in the continued presence of the indicated barbiturates. Results are expressed as percent of control. In these experiments, control [^{3}H]-dGlc uptakes were 2.67 ± 0.44 pmol/15 min∗μg cellular protein. The means of individual experiments (N = 3) are presented. Each data set is normalized to its respective control. Error bars are ±SD. The oxy-barbiturates (*top panel*) were secobarbital (▲), pentobarbital (●), phenobarbital (○), barbital (■), and barbituric acid (□). Results for methohexital, amobarbital, and allobarbital are excluded from the plot for clarity but the IC_{50}s are reported in Table 1. The thio-barbiturates (*bottom panel*) were thiamylal (●), thiobutabarbital (○), thiopental (■), and thiobarbituric acid (□). The lines are meant to guide the eye of the reader.

respectively (*not shown*). In contrast, slightly lowering the buffer pH to 6.8 produces no analogous change in the IC_{50} for pentobarbital (i.e., 0.7 mM and 0.8 mM at pH 6.8 and pH 7.4, respectively) (*not shown*). As expected (Franks & Lieb, 1994), this pH dependence predicts that it is the neutral form, as opposed to the anionic form, of the barbiturate which elicits inhibition. Additionally, experiments with the (+) and (−) optical isomers of pentobarbital demonstrate no clear stereospecificity for inhibition of Glc transport by barbiturates (*not shown*). Analogous, pharmacologic characterization of the effects of barbiturates on GLUT-1-mediated Glc transport in human erythrocytes, i.e., unidirectional Glc flux, yielded essentially similar findings (El-Barbary et al., 1996; and *not shown*).

In contrast to the rather robust inhibition observed for barbiturates, none of the several halogenated hydrocarbon anesthetics, such as halothane (Fig. 1), examined significantly inhibit Glc transport in 3T3-C2 cells at pharmacologically relevant concentrations (i.e., ≤10 mM) (Table 1). In a likewise manner, ethanol was also unable to significantly inhibit Glc transport (Table 1) at pharmacologically relevant concentrations (i.e., ≤10 mM). Inhibition of GLUT-1 mediated Glc transport by ethanol has, however, been observed at suprapharmacological concentrations (Kutchai, Chandler & Geddis, 1980; Krauss, Diamond & Gordon, 1994, and *not shown*). Additionally, the halogenated hydrocarbons and ethanol had no significant effect on GLUT-1-mediated Glc transport, i.e., unidirectional Glc flux, in human erythrocytes (Honkanen et al., 1995; El-Barbary et al., 1996, and *not shown*).

Table 1. Anesthetic potencies (IC_{50}s) for inhibition of Glc transport in 3T3-C2 murine fibroblasts*

Class	Anesthetic	IC_{50} (mM)
Oxy-barbiturates	Secobarbital	0.4
	Amobarbital	0.8
	Pentobarbital	0.8
	Methohexital	0.9
	Phenobarbital	1.7
	Barbital	2.1
	Allobarbital	2.9
	Barbituric Acid	>10
Thio-barbiturates	Thiamylal	0.2
	Thiobutabarbital	1
	Thiopental	2
	Thiobarbituric acid	>10
Halogenated hydrocarbons†	Desflurane	>10
	Enflurane	>10
	Halothane	>10
	Isoflurane	>10
	Methoxyflurane	>10
Other†	Ethanol	>10

*Fibroblasts were treated with the anesthetics (0–10 mM) and Glc transport was assayed as described in the legend to Fig. 2. The IC_{50}s were calculated from the parameters obtained by computer analysis to nonlinear regressions utilizing a four parameter logistic function that does not make any assumptions regarding maximal inhibition.
†Inhibition of Glc transport by all of the volatile anesthetics (≤10 mM) is ≤15% of control. Solutions of volatile anesthetics were prepared daily and stored in sealed glass containers. Plates of fibroblasts were kept in tight-fitting sealed plastic bags during anesthetic pretreatment and assay.

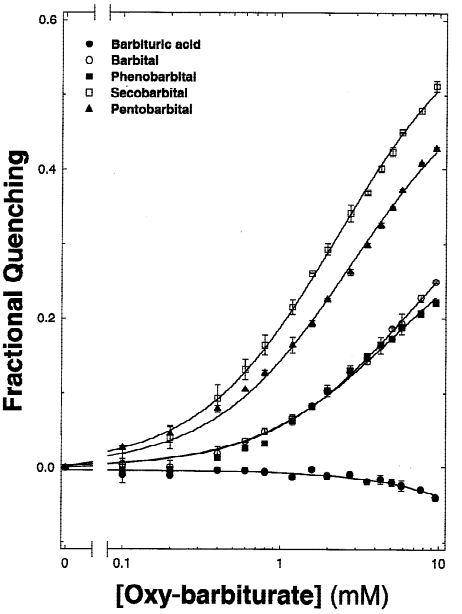


Fig. 3. Oxy-barbiturates interact directly with purified GLUT-1. Purified and reconstituted GLUT-1 (~25 μg/ml) in FB was incubated with different concentrations (0–10 mM) of barbituric acid (●), barbital (○), phenobarbital (■), secobarbital (□), or pentobarbital (▲) as indicated and fractional quenching (*FQ*) calculated as described in Materials and Methods. The mean of three separate experiments each consisting of two titrated samples are depicted and the error bars are ±SD. The curves were computer fit to simple hyperbolic functions of saturable oxy-barbiturate binding except for the barbituric acid curve (●) which is not mathematically derived and is simply drawn to guide the eye of the reader. The fits were used to obtain the apparent K_ds reported in the text of the Results.

Several Oxy-Barbiturates, but not Halothane or Isoflurane, Interact Directly with Purified GLUT-1

We have previously demonstrated (Honkanen et al., 1995) that pentobarbital quenches the intrinsic protein fluorescence of purified GLUT-1 in a concentration-dependent and saturable manner which is inconsistent with simple collisional quenching (Lakowicz, 1983). From this finding we concluded that barbiturates are able to interact directly with, i.e., bind to, GLUT-1 protein. The concentration dependence for several oxy-barbiturates (0–10 mM) of the quenching of the intrinsic protein fluorescence of purified GLUT-1 was examined (Fig. 3). All of the oxy-barbiturates examined, except barbituric acid, quench the intrinsic fluorescence of GLUT-1 in a saturable manner. The apparent K_ds for this interaction of secobarbital, pentobarbital, phenobarbital, barbital, and barbituric acid are 2.3, 2.8, 5.6, 6.5, and >10 mM. This hierarchy of apparent affinities of oxy-barbiturates for GLUT-1 parallels that of the IC_{50}s for inhibition of Glc transport. The results are also consistent with the Meyer-Overton rule (Franks & Lieb, 1994) and validate that, like inhibition of Glc transport, the direct interaction of barbiturates with GLUT-1 requires the presence of substituents at R_3, R_{5a}, and/or R_{5b} on the core structure (Fig. 1). We were unable to perform similar experiments with thio-barbiturates because these anesthetics absorb ultraviolet light greatly and therefore interfere with this fluorescence quenching approach.

The concentration dependence for halothane and isoflurane (0–10 mM) of the quenching of the intrinsic protein fluorescence of purified GLUT-1 was also examined (*not shown*). Halothane and isoflurane both efficiently quench the intrinsic fluorescence of GLUT-1 but the process is generally linear and clearly not saturable. This result is most consistent with simple collisional quenching (Lakowicz, 1983) and qualitatively resembles the linear concentration dependance for halothane and isoflurane quenching of the intrinsic fluorescence of Trp (1 μM, *not shown*). As a positive control for our ability to observe the direct interaction of volatile halogenated hydrocarbon anesthetics with putative binding sites on proteins, we examined the concentration dependance for halothane (0–10 mM) of the quenching of the intrinsic protein fluorescence of bovine serum albumin (20 μg/ml,

not shown). Consistent with the findings of Eckenhoff et al. (1997), we observed that the interaction of halothane with bovine serum albumin is a saturable process (K_d ~1.8 mM). Thus, oxy-barbiturates, but not halothane or isoflurane, interact directly with purified and reconstituted GLUT-1.

Barbiturates Differentially Inhibit Glc Transport Mediated by Several Facilitative Glc Transporter Isoforms

The concentration dependence for the inhibition of [^{3}H]-dGlc and [^{14}C]-MeGlc uptake by pentobarbital in cell systems expressing different isoforms of the facilitative Glc transporters varies (Fig. 4). 3T3-C2 murine fibroblasts expressing predominantly GLUT-1 were used as a model for this isoform. G6 AtT20ins cells expressing predominantly GLUT-2 were used as a model for this isoform. Rat cerebellar neurons expressing predominantly GLUT-3 were used as a model for this isoform. Insulin-stimulated rat epididymal white adipocytes expressing predominantly GLUT-4 were used as a model for this isoform.

Pentobarbital strongly inhibits [^{3}H]-dGlc uptake (Fig. 4, *top panel*) by three of the facilitative Glc transporters examined, i.e., GLUT-1, GLUT-2, and GLUT-3, but only weakly inhibits GLUT-4. The IC_{50}s for inhibition of [^{3}H]-dGlc uptake by pentobarbital are presented in Table 2. Pentobarbital inhibited GLUT-1, GLUT-2, and GLUT-3 with IC_{50}s of ~1 mM. For GLUT-1 similar results for the sensitivity to pentobarbital (IC_{50} of ~1 mM) of unidirectional Glc flux were observed in human erythrocytes (El-Barbary et al., 1996; and *not shown*). For GLUT-2 similar results were obtained for the inhibition by pentobarbital (IC_{50} of ~1 mM) of Glc transport in G2 AtT20ins cells (*not shown*). These cells are a sister clone with a very similar phenotype to G6 AtT20ins cells (Hughes et al., 1988). Nearly all of the Glc transport by insulin-stimulated rat epididymal white adipocytes is mediated by GLUT-4 (Baldwin, 1993) yet this system shows only very modest sensitivity (IC_{50} of ~10 mM) to pentobarbital relative to GLUT-1. Glc transport by insulin-stimulated 3T3-L1 adipocytes is also less sensitive to inhibition by pentobarbital (IC_{50} of ~3 mM, *not shown*) than 3T3-C2 fibroblasts (IC_{50} of ~0.8 mM). Additionally, undifferentiated 3T3-L1 fibroblasts and 3T3-L1 adipocytes not stimulated by insulin exhibited IC_{50}s of ~1.0 and 1.8 mM, respectively. The difference between these results and those presented for pentobarbital inhibition of Glc transport in insulin-stimulated rat adipocytes (IC_{50} of >10 mM) is probably attributable to the expression of significantly more functional GLUT-1, relative to GLUT-4, in insulin-stimulated 3T3-L1 adipocytes and some GLUT-4 being functionally expressed by 3T3-L1 adipocytes in the basal state (Gould & Holman, 1993).

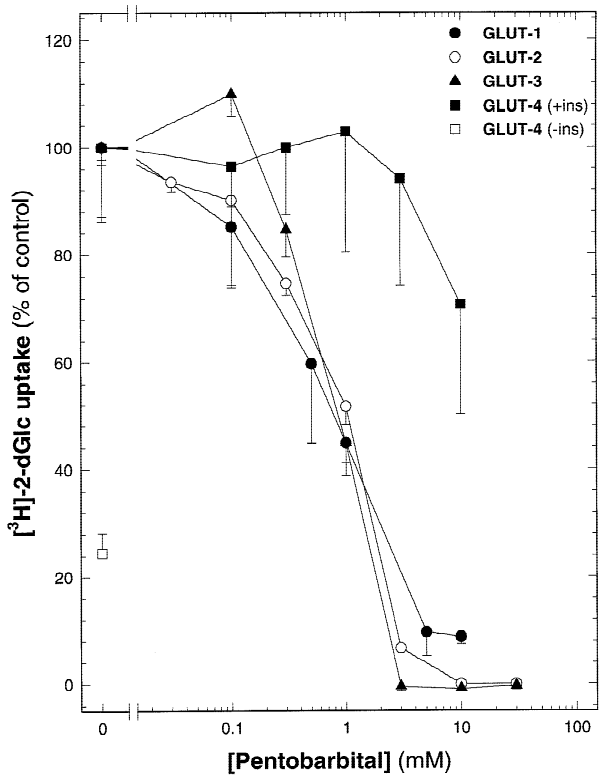
We have previously established (Honkanen et al., 1995) that barbiturates are directly inhibiting GLUT-1 mediated Glc transport and that inhibition is not by virtue of metabolic alterations. The concentration dependence for the inhibition by pentobarbital of [^{14}C]-MeGlc uptake by rat neurons, G6 AtT20ins cells, and rat adipocytes, like [^{3}H]-dGlc uptake, also varies (Fig. 4, *bottom panel*). Pentobarbital inhibits [^{14}C]-MeGlc uptake mediated by GLUT-3 (IC_{50} of ~1 mM) and GLUT-2 (IC_{50} of ~1 mM). GLUT-4 (IC_{50} of >10 mM) is much less sensitive to inhibition by pentobarbital. These findings validate the [^{3}H]-dGlc uptake results. MeGlc is a non-metabolizable substrate for Glc transport (Carruthers, 1990). This suggests that, as for GLUT-1, inhibition by barbiturates of GLUT-2 and GLUT-3 mediated glucose transport does not involve metabolic alterations.

A potentially trivial explanation for the insensitivity of Glc transport to barbiturate inhibition in rat adipocytes is hydrophobic partitioning of the drug into the lipid stores of these cells. In this way, the barbiturate might be unavailable for interactions with GLUT-4. Given the partition coefficient of pentobarbital from buffered saline (pH 7) into erythrocyte membranes at 23° is ~12.7 (Firestone et al., 1986) and the volume of adipocytes in the assay is ~15% it seems that at 10 mM pentobarbital it would be unlikely that a large fraction of the total pentobarbital would be removed from the bulk aqueous phase into the lipid stores of rat adipocytes. Additionally, even if some partitioning does occur, the drug is presumably in equilibrium between the buffer, lipid stores, membrane bilayers, and membrane proteins of the adipocytes and thus should be available for interactions with GLUT-4. We have, however, tested this question experimentally (*not shown*) by preincubating (15 min) buffers containing appropriate concentrations of barbiturate (1 and 10 mM pentobarbital) with adipocytes (15% vol/vol) and subsequently applying these adipocyte-treated buffers containing barbiturates to murine fibroblasts. This pretreatment of the buffers with adipocytes does not significantly attenuate the observed inhibition of Glc transport by both maximal (10 mM) and submaximal (1 mM) concentrations of pentobarbital in murine fibroblasts (*not shown*). We believe that this demonstrates that potential hydrophobic partitioning of drug into the lipid stores of the adipocytes does not adequately explain the lower sensitivity to barbiturate inhibition of GLUT-4 mediated Glc transport in insulin-stimulated rat adipocytes.

Discussion

Pharmacologic Specificity

The observation that barbiturates, but not halogenated hydrocarbons, can significantly inhibit GLUT-1 medi-



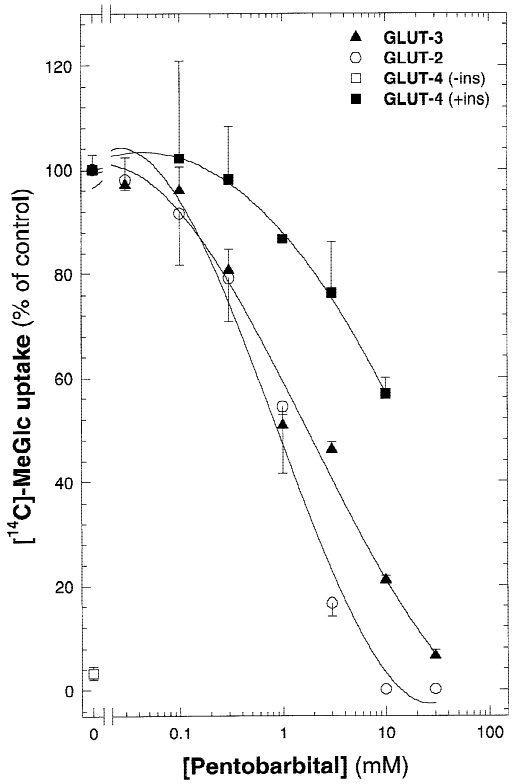


Table 2. IC_{50}s for the inhibition of Glc transport by pentobarbital in cell systems expressing different isoforms of the facilitative Glc transporters*

Facilitative Glc Transporter Isoform	Cell System	IC_{50} for Inhibition of Glc Transport by Pentobarbital (mM)
GLUT-1	3T3-C2 cells	0.8
GLUT-2	G6 AtT20ins cells	1.0
GLUT-3	Rat cerebellar neurons	0.7
GLUT-4	Insulin-stimulated rat adipocytes	>10

*The indicated cell systems were treated with pentobarbital (0–10 mM) and Glc transport assayed as described in the legend to Fig. 4. The IC_{50}s were calculated from the parameters obtained by computer analysis to nonlinear regressions utilizing a four parameter logistic function that does not make any assumptions regarding maximal inhibition.

ated Glc transport suggests specificity with respect to anesthetic class (Fig. 2, Table 1). We have demonstrated (Honkanen et al., 1995; Fig. 3) that barbiturates interact directly with GLUT-1. The present results also indicate that halogenated hydrocarbons may not interact directly with GLUT-1. The specific targets of both of these anesthetic classes, i.e., barbiturates and halogenated hydrocarbons, are not fully elucidated (Harvey, 1985; Firestone et al., 1986; Franks & Lieb, 1994). Both of these structurally distinct (Fig. 1) classes have been hypoth-

Fig. 4. Concentration dependence for inhibition of Glc transport by pentobarbital in cell systems expressing different isoforms of the facilitative Glc transporters. The effect of the indicated concentrations of pentobarbital on [^{3}H]-dGlc uptake (*top panel*) by 3T3-C2 murine fibroblasts (GLUT-1) (●) was assayed in triplicate at 23°C as described by Honkanen et al. (1995). Control [^{3}H]-dGlc uptakes were 2.3 pmol/15 min∗μg cellular protein. The effect of the indicated concentrations of pentobarbital on [^{3}H]-dGlc uptake (*top panel*) and [^{14}C]-MeGlc uptake (*bottom panel*) by G6 AtT20ins cells (GLUT-2) (○) was assayed in triplicate at 23°C as described by Maher et al. (1996). Control [^{3}H]-dGlc and [^{14}C]-MeGlc uptakes were 5.3 pmol/min∗10^6 cells and 1.4 pmol/min∗10^6 cells, respectively. The effect of the indicated concentrations of pentobarbital on [^{3}H]-dGlc uptake (*top panel*) and [^{14}C]-MeGlc uptake (*bottom panel*) by rat cerebellar neurons (GLUT-3) (▲) was assayed in triplicate at 23°C as described by Maher et al. (1996). Control [^{3}H]-dGlc and [^{14}C]-MeGlc uptakes were 2.8 pmol/min∗10^6 cells and 1.1 pmol/min∗10^6 cells, respectively. The effect of the indicated concentrations of pentobarbital on [^{3}H]-dGlc uptake (*top panel*) and [^{14}C]-MeGlc uptake (*bottom panel*) by insulin-stimulated (■) rat adipocytes (GLUT-4) was assayed in triplicate at 23°C as described by Weber et al. (1988). Control [^{3}H]-dGlc and [^{14}C]-MeGlc uptakes were 145 pmol/min∗10^6 cells and 3.3 pmol/min∗10^6 cells, respectively. The uptake in the absence of insulin (i.e., basal) (□) is shown for comparison. Results are expressed as percent of control and the means of individual experiments ($N = 3$) are presented. Each data set is normalized to its respective control. Error bars are ±SD. The lines are meant to guide the eye of the reader.

esized to have direct and/or indirect effects on γ-amino butyric acid (GABA) receptors, Ca^{++} channels, and ion pumps (Harvey, 1985; Firestone et al., 1986; Franks & Lieb, 1994). Clearly GLUT-1 constitutes a novel target for barbiturates which seems to not be influenced by halogenated hydrocarbons. This suggests that because of Glc transport inhibition there may be unanticipated metabolic consequences of barbiturate administration that are not elicited by the halogenated hydrocarbons. Elegant physiologic studies by Warner and coworkers (e.g., Nakashima, Todd & Warner, 1995) illustrate the complexities of these questions with regard to the roles of cerebral metabolic rate, membrane depolarization, and potential neuroprotection. These effects might have a positive and/or negative impact on the many clinical uses of these anesthetic agents.

Isoform Specificity

It is interesting to speculate why GLUT-4, but not the other isoforms of the facilitative Glc transporters, is relatively insensitive to inhibition by barbiturates (Fig. 4, Table 2). It is perhaps reasonable, based upon our studies with purified GLUT-1 (Honkanen et al., 1995; Fig. 3), to presume that barbiturates may directly interact with GLUT-1, GLUT-2, and GLUT-3. Also, that any direct interaction of barbiturates with GLUT-4 is probably of lower affinity. Is there a particular structural feature of GLUT-4 that is different from the other isoforms which might make it less sensitive to barbiturates? All of the isoforms examined in this study are ~60% identical with respect to amino acid sequence and have been predicted or demonstrated to display approximately the same transmembrane topologies (Baldwin, 1993). In fact, the functional intramembranous domains or Glc transport machinery are even less structurally divergent than the extramembranous portions. There is, therefore, no obvious structural feature of GLUT-4, relative to the other isoforms, which is a likely candidate for imparting it with less sensitivity to barbiturates. Of course this does not preclude a subtle structural difference among these Glc transporter isoforms from explaining this differential sensitivity to barbiturates.

Our recent demonstration in human erythrocytes (El-Barbary et al., 1996) that GLUT-1-mediated equilibrium exchange of sugars is less sensitive to inhibition by barbiturates than unidirectional sugar flux suggests another possibility. Namely, some subtle feature of the carrier responsible for its apparent kinetic asymmetry (Carruthers, 1990) might impart it with sensitivity to barbiturates. Consistent with this thinking is the observed lack of kinetic asymmetry for GLUT-4 (Gould & Holman, 1993). The recent demonstration by Maher et al. (1996), utilizing a combination of results obtained in studies of GLUT-3-mediated Glc transport kinetics in neonatal rat cerebellar neurons and *Xenopus laevis* oocytes, that GLUT-3 is kinetically asymmetric is also in agreement with this thinking. Like GLUT-4, GLUT-2 in isolated rat hepatocytes has been reported in an earlier study (Craik & Elliot, 1979) to be kinetically symmetrical. This finding has, however, not been independently confirmed (*see review* of Carruthers, 1990), and there are still clear ambiguities regarding the kinetic properties of GLUT-2. In light of these observations, it seems that kinetic asymmetry may not be the appropriate explanation for the diminished sensitivity of GLUT-4 to barbiturates. This issue does, however, warrant further investigation.

The selectivity and affinity of the different facilitative Glc transporter isoforms for a variety of substrates (i.e., Glc, dGlc, Gal, Fru, etc.) and inhibitors (i.e., cytochalasin B, forskolin, phloretin, Hg^{++}, etc.) show subtle but clear heterogeneity (*for general reviews, see* Carruthers, 1990; Baldwin, 1993; Gould & Holman, 1993; Maher et al., 1994; Vannucci et al., 1997). Some of these distinctions have been molecularly mapped by the engineering of chimeric transporters containing different portions of two different isoforms in heterologous expression systems (i.e., *X. laevis* oocytes and Chinese hamster ovary cells) (e.g., Asano et al., 1992; Buchs et al., 1995; Arbuckle et al., 1996). Thus, it seems reasonable to assume that subtle structural features of GLUT-4 may impart it with lowered sensitivity, relative to GLUT-1, to inhibition by barbiturates. This seems to be the first clear instance in which a ligand (i.e., substrate or inhibitor) interacts preferentially (i.e., higher affinity) with GLUT-1, GLUT-2, and GLUT-3 relative to GLUT-4. The only other property of GLUT-4 that clearly distinguishes it from these other three isoforms is its ability to be recruited to the plasma membrane in response to insulin (Baldwin, 1993). Any potential relationship between these two properties of GLUT-4, namely insulin-induced translocation and insensitivity to barbiturates, is not evident. It will be interesting to examine chimeras of GLUT-1 and GLUT-4 with respect to barbiturate sensitivity and attempt to map this putative barbiturate inhibition domain.

Conclusions

We have demonstrated that: (i) GLUT-1 is functionally sensitive to inhibition by a wide variety of oxy- and thio-barbiturates but not halogenated hydrocarbons or ethanol (at $\leq$10 mM); (ii) Inhibition of GLUT-1-mediated Glc transport by barbiturates requires hydrophobic side chains on the core structure; (iii) Barbiturates, but not halogenated hydrocarbon anesthetics, interact directly with GLUT-1 and the characteristics of this interaction parallel those of Glc transport inhibition; (iv) GLUT-1, GLUT-2, and GLUT-3 are much more

sensitive than GLUT-4 to inhibition by barbiturates; and (iv) Potential barbiturate-induced metabolic changes mediated by inhibition of Glc transport, as compared to the use of halogenated hydrocarbon inhalation anesthetics, should be further evaluated.

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