Monocarboxylate Transporter-Mediated Transport of γ -Hydroxybutyric Acid in Human Intestinal Caco-2 Cells

Wing Ki Lam, Melanie A. Felmler, and Marilyn E. Morris

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Amherst, New York

Received October 13, 2009; accepted December 1, 2009

ABSTRACT:

The objectives of this study were to determine mRNA expression of monocarboxylate transporters (MCT) and to evaluate intestinal transport of the MCT substrates γ -hydroxybutyrate (GHB) and D-lactate in human intestinal Caco-2 cells. The presence of mRNA for MCT1, 2, 3, and 4 was observed in Caco-2 cells. The uptake of both GHB and D-lactate in Caco-2 cells was demonstrated to be pH- and concentration-dependent and sodium-independent. The uptake of GHB and D-lactate was best described by a Michaelis-Menten equation with passive diffusion (GHB: $K_m = 17.6 \pm 10.5$ mM, $V_{max} = 17.3 \pm 11.7$ nmol/min/mg, and $P = 0.38 \pm 0.15$ μ l/min/mg; and D-lactate: $K_m = 6.0 \pm 2.9$ mM, $V_{max} = 35.0 \pm 18.4$ nmol/min/mg, and

$P = 1.3 \pm 0.6$ μ l/min/mg). The uptake of GHB and D-lactate was significantly decreased by the known MCT inhibitor α -cyano-4-hydroxycinnamate and the MCT substrates GHB and D-lactate but not by the organic cation tetraethylammonium chloride. Directional flux studies with both GHB and D-lactate suggested the involvement of carrier-mediated transport with the permeability in the apical to basolateral direction higher than that in the basolateral to apical direction. These findings confirm the presence of MCT1-4 in Caco-2 cells and demonstrate GHB and D-lactate transport characteristics consistent with proton-dependent MCT-mediated transport.

Monocarboxylic acid transporters (MCTs), members of the SLC16A family, are proton-linked transporters that play a crucial role in cellular metabolism. To date 14 MCT-related sequences have been identified in mammals through sequence homology; however, only seven isoforms have been functionally characterized (Halestrap and Meredith, 2004; Murakami et al., 2005). These isoforms differ in terms of tissue distribution, substrate specificities, and affinities with only four isoforms (MCT1-4) characterized as proton-dependent monocarboxylate transporters (Halestrap and Meredith, 2004; Bonen et al., 2006).

MCT1 is ubiquitously expressed in human tissues; however, specific tissue localization (apical versus basolateral membrane) varies (Halestrap and Price, 1999). MCT2 (60% homology with MCT1) demonstrates a more restricted distribution with the greatest expression observed in the testis (Lin et al., 1998). In contrast to MCT1, multiple MCT2 transcripts are observed in humans, suggesting the occurrence of pretranslational regulation (Lin et al., 1998). MCT3 demonstrates the most restricted tissue distribution with expression in the basolateral membrane of the retinal pigment epithelium (Philp et

al., 2003); however, recent studies have demonstrated MCT3 mRNA expression in smooth muscle cell lines and human aorta (Zhu et al., 2005). MCT4, which is most closely related to MCT1 in terms of tissue distribution and regulation, is predominantly expressed in cells with a high glycolytic rate (such as tumor, muscle, and white blood cells), where it is involved in the removal of lactic acid produced from glycolysis (Juel and Halestrap, 1999; Manning Fox et al., 2000).

MCT1-4 have been demonstrated to transport a wide range of endogenous and exogenous compounds including lactate, butyrate, pyruvate, γ -hydroxybutyric acid (GHB), pravastatin, simvastatin, XP13512, and carindacillin (Morris and Felmler, 2008). However, the specific isoforms vary in their substrate specificity and affinity, as well as in their response to inhibitors. MCT1 and MCT2 have very similar substrate specificities, but they differ with respect to affinity. MCT2 is a high-affinity pyruvate transporter demonstrating a 100-fold greater affinity than that for MCT1 (Lin et al., 1998). In addition, MCT1 and MCT2 can be distinguished by their sensitivity to inhibitors; MCT2 is not inhibited by *p*-chloromercuribenzenesulfonic acid, a known MCT1 inhibitor (Halestrap and Meredith, 2004). In contrast to MCT2, MCT4 demonstrates lower affinities for monocarboxylates and is sensitive to *p*-chloromercuribenzenesulfonic acid-mediated inhibition (Morris and Felmler, 2008). MCT1 and MCT4 require a different ancillary protein than does MCT2 (CD147 versus EMBIGIN), which represents a potential mechanism contributing to the observed variation in inhibitor sensitivities (Halestrap and Meredith, 2004).

In this investigation we characterized the transport of two known

This work was supported in part by the National Institutes of Health National Institute on Drug Abuse [Grant DA023223]. W.K.L. was supported in part by an Undergraduate Summer Fellowship, and M.A.F. was supported by a Graduate Fellowship from Pfizer Global Research and Development.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.109.030775.

ABBREVIATIONS: MCT, monocarboxylate transporter; GHB, γ -hydroxybutyric acid; XP13512, ($\pm$)-1-([(α -isobutanoyloxyethoxy)carbonyl] amino-methyl)-1-cyclohexane acetic acid; CHC, α -cyano-4-hydroxycinnamate; TEA, tetraethylammonium chloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; AP, apical; BL, basolateral; SMCT, sodium-coupled monocarboxylate transporter; siRNA, small interfering RNA.

MCT substrates, D-lactate and GHB, a drug of abuse. Clinically, GHB is approved for the treatment of narcoleptic patients with cataplexy, marketed under the brand name of Xyrem (Scharf et al., 1998), and for the treatment of alcohol withdrawal syndrome (Poldrugo and Ad-dolorato, 1999). GHB exhibits nonlinear pharmacokinetics in rats (Lettieri and Fung, 1979) and humans (Palatini et al., 1993) involving capacity-limited absorption (Arena and Fung, 1980), metabolism (Lettieri and Fung, 1979; Palatini et al., 1993), and renal elimination (Morris et al., 2005). The roles of MCTs in the renal transport and clearance of GHB have been demonstrated (Wang and Morris, 2007), but the importance of MCTs in the intestinal absorption of GHB has not been investigated. MCTs are expressed throughout the intestine with the highest expression levels in the distal colon (Gill et al., 2005). MCT1 is the primary isoform expressed in the intestine with localization reported at both the apical (Cuff et al., 2002; Gill et al., 2005) and basolateral (Iwanaga et al., 2006) membranes. In this study, Caco-2 cells (human colonic cell line), a widely accepted in vitro model for drug absorption and metabolism studies (Shah et al., 2006), were used to characterize the intestinal transport of GHB. Caco-2 cells express mRNA levels of MCT1 similar to those reported in human ileum and jejunum, although levels are less than that observed in human colon (Englund et al., 2006). Studies examining mRNA and protein expression of MCT1, as well as studies with apical membrane vesicles isolated from Caco-2 cells and transport studies with Caco-2 monolayers have all reported the apical localization of MCT1 (Hadjagapiou et al., 2000; Buyse et al., 2002; Cuff et al., 2002; Konishi et al., 2003). The objectives of this study were 1) to evaluate the expression of multiple MCT isoforms in Caco-2 cells, 2) to characterize the driving forces of GHB and D-lactate uptake in Caco-2 cells by determining the effects of pH, sodium, concentration, and MCT inhibitors on uptake, and 3) to determine the directional flux of GHB and D-lactate across Caco-2 cells.

Materials and Methods

Chemical and Reagents. Minimal essential medium, antibiotic-antimycotic solution (100×), fetal bovine serum, and Hanks' balanced salt solution were obtained from Invitrogen (Carlsbad, CA). Sodium GHB, lithium D-lactate, α -cyano-4-hydroxycinnamate (CHC), tetraethylammonium chloride (TEA), HEPES, and MES were obtained from Sigma-Aldrich (St. Louis, MO). [3 H]GHB (50 Ci/mmol) was purchased from Moravak Biochemicals (Brea, CA), and [3 H]D-lactate (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Biodegradable counting scintillant was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 cells were grown in T-75 culture flasks with fresh culture medium replaced every other day. The culture medium consisted of minimal essential medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Invitrogen). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂/95% air. Confluent Caco-2 cells were subcultured using 0.25% trypsin-EDTA in a ratio of 1:3 for T-75 culture flasks or 1:12 for 35-mm² culture dishes or Transwell inserts. For all of the uptake studies, Caco-2 cells were seeded on 35-mm² culture dishes and used

for experiments 2 days after seeding. For the directional flux study, Caco-2 cells were seeded on Transwell polycarbonate membrane inserts (Corning Inc., Corning, NY) for 25 days with fresh medium replaced every other day for the first 10 days and every day for the next 15 days to ensure the complete formation of monolayers with tight junctions. All of the cells used in this study were between passage numbers 45 and 60. No changes in MCT1 expression in Caco-2 cells with culture time have been reported (Englund et al., 2006).

RT-PCR. RT-PCR analysis of MCT1-4 was performed as described previously (Wang et al., 2006a,b). In brief, total RNA was isolated from Caco-2 cells with the SV Total RNA Isolation System (Promega, Madison, WI). First-strand cDNA was synthesized from 2 μ g of total RNA by reverse transcriptase using oligo(dT) primers and a Brilliant Two-Step QRT-PCR core reagent kit (Agilent Technologies, Cedar Creek, TX). An Eppendorf Mastercycler gradient PCR system was used to perform the PCR. Primers specific to human MCT1-4 (Table 1) were designed using the Primer Express software program and have been used previously to examine MCT expression in human kidney and human kidney HK-2 cells (Wang et al., 2006b). The PCR reaction mixtures contained 200 μ M concentrations of each deoxynucleotide triphosphate, 0.2 μ M concentrations of each forward and reverse primer, and 0.5 unit/reaction Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA) in 1× PCR buffer [500 mM KCl, 100 mM Tris-HCl, pH 8.3 (at 25°C), and 15 mM magnesium] through 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel, stained by ethidium bromide, and visualized under UV light.

Transport Studies. Caco-2 cells grown in 35-mm² culture dishes for 2 days were incubated with assay buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM MES, pH 6.0) for time- and concentration-dependent studies. For pH-dependent uptake studies, cells were incubated with assay buffer at different pH values ranging from pH 5.5 to 7.5. For sodium-dependent studies, cells were washed with assay buffer containing sodium (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, pH 7.5) or N-methyl-D-glucamine (137 mM N-methyl-D-glucamine, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, pH 7.5). Uptake was initiated by the addition of 1 ml of assay buffer containing [3 H]GHB or [3 H]D-lactate, and cells were incubated for 5 min at room temperature. Substrate uptake was stopped by rinsing cells three times with ice-cold buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM HEPES, pH 7.5). One milliliter of lysis buffer containing 0.3 N NaOH and 1% SDS was added to solubilize the cells, and the radioactivity present in 200- μ l aliquots was determined by liquid scintillation counting (1900 CA, Tri-Carb Liquid Scintillation Analyzer; PerkinElmer Life and Analytical Sciences, Waltham, MA). CHC (2 mM), a known MCT inhibitor, was used to inhibit MCT-mediated transport. TEA (5 mM), a known inhibitor of organic cation transporters, was used as a negative control. The protein content was determined with a BCA assay kit (Thermo Fisher Scientific) following the manufacturer's instructions.

GHB and D-lactate uptake kinetics were evaluated using simple Michaelis-Menten (eq. 1) or atypical kinetics (including multiple transporters and/or a diffusional component) (eqs. 2–4) after initial Eadie-Hofstee plot evaluation. Transport kinetic parameters were evaluated by nonlinear regression analysis (WinNonlin version 5.1; Pharsight, Mountain View, CA). The model of best fit was selected based on a visual inspection of fits and a comparison of Akaike Information Criterion Values.

TABLE 1
Primers used for RT-PCR analysis

Isoform	Accession No.	Direction	Sequences	Product
MCT1	NM_003051	Forward	5'-TGGATGGAGAGGAAGCTTTCTAAT-3'	151
		Reverse	5'-CACACCAGATTTTCCAGCTTTC-3'	
MCT2	NM_004731	Forward	5'-GCCCCACTGGCACAGGACTA-3'	251
		Reverse	5'-CACAATAGCCCCACAGACAT-3'	
MCT3	NM_013356	Forward	5'-GGATGCGTTGAAGAACTATGAGATC-3'	213
		Reverse	5'-CCGGGTTCTCTCTGCAACA-3'	
MCT4	NM_004207	Forward	5'-CACGGCATCGTCACCAACT-3'	200
		Reverse	5'-ACAGCCTGGATAGCAACGTACAT-3'	

$$v = \frac{V_{\max} \cdot C}{K_m + C} \quad (1)$$

$$v = \frac{V_{\max} \cdot C}{K_m + C} + P \cdot C \quad (2)$$

$$v = \frac{V_{\max 1} \cdot C}{K_{m1} + C} + \frac{V_{\max 2} \cdot C}{K_{m2} + C} \quad (3)$$

$$v = \frac{V_{\max 1} \cdot C}{K_{m1} + C} + \frac{V_{\max 2} \cdot C}{K_{m2} + C} + P \cdot C \quad (4)$$

Directional Flux Studies. Caco-2 cells were prepared for the directional flux study by culturing cells on Transwell permeable supports for 25 days, with transport studies conducted using either [^3H]GHB or [^3H]D-lactate. The studies were performed as described by Zhang and Morris (2003). In brief, Caco-2 cells on Transwell permeable supports with transepithelial electrical resistance values higher than $500 \Omega \cdot \text{cm}^2$ were washed with transport buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl_2 , 1.2 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5% glucose, and 10 mM MES for pH 6.0 buffer or 10 mM HEPES for pH 7.5 buffer) three times at 37°C . Transport buffer containing radiolabeled substrate was added to the apical (AP) (1.5 ml) or basolateral (BL) (2.6 ml) chamber, whereas nonradio-labeled transport buffer was added to the opposite chamber. Samples were incubated at 37°C and 100- μl aliquots were taken from the receiver side at 30, 60, and 90 min for liquid scintillation counting (Wang et al., 2006b).

Fig. 1. mRNA expression of MCT1-4 in Caco-2 cells. PCR products [151 base pairs (bp) for MCT1, 251 bp for MCT2, 213 bp for MCT3, and 200 bp for MCT4] were separated on a 2% agarose gel.

The apparent permeability coefficients (P_{app}) were calculated for the directional flux studies using eq. 5 (Zhang and Morris, 2003):

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{C_0 \cdot A} \quad (5)$$

where $\Delta Q/\Delta t$ is the rate of the appearance of radiolabeled substrates in the receiver chamber, C_0 is the initial concentration of the radiolabeled compound in the donor chamber, and A is the surface area of the insert (4.71 cm^2).

Statistical Analysis. Data analysis was performed using GraphPad Prism (version 4.0, GraphPad Software Inc., San Diego, CA). Significant differences between means were determined by one-way analysis of variance followed by a Dunnett's post hoc test or a two-way analysis of variance with a Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.

Results

MCT Expression in Caco-2 Cells. In agreement with previous studies (Hadjigapiou et al., 2000; Lecona et al., 2008), expression of MCT1, MCT3, and MCT4 mRNA was detected in Caco-2 cells using isoform-specific primers (Table 1; Fig. 1). Our study also demonstrated mRNA expression of MCT2 in Caco-2, which was not observed or assessed in previous studies.

[^3H]GHB and D-Lactate Uptake Studies. Preliminary studies demonstrated that GHB and D-lactate uptake was linear up to 10 min (data not shown), and an incubation time of 5 min was selected for all subsequent uptake studies. The effect of pH on the uptake of GHB and D-lactate in Caco-2 cells was evaluated by incubating [^3H]GHB or [^3H]D-lactate at pH values ranging from pH 5.5 to 7.5 (Fig. 2, A and B). The uptake rates of GHB and D-lactate increased with decreasing pH with significantly higher uptake rates observed at pH 5.5, 6.0, and 6.5 (D-lactate only) compared with control (pH 7.5). The pH-dependent nature of GHB transport suggests the involvement of a proton-coupled transport system such as MCTs. A pH of 6.0 was selected for the concentration-dependent and directional flux studies because this pH is more clinically relevant than a lower pH value of 5.5 and to minimize the changes in the ionization state of GHB that could possibly influence diffusion. In contrast, incubation of GHB and D-lactate with and without sodium

Fig. 2. Driving forces of GHB uptake in Caco-2 cells. Influence of pH on GHB (0.1 mM) (A) and D-lactate (0.1 mM) (B) uptake. Data are expressed as a percentage of control (pH 7.5). Influence of sodium on GHB (0.1 mM) (C) and D-lactate (0.1 mM) (D) uptake. Data are expressed as percentage of control (without sodium). Results are presented as mean $\pm$ S.E.M. for three independent experiments performed in triplicate. *, $P < 0.05$ compared with control.

resulted in no difference in the rate of uptake (Fig. 2, C and D), indicating that sodium-coupled monocarboxylate transporters (SMCTs) are not involved in the transport of GHB in Caco-2 cells.

Concentration-Dependent Transport Studies. The concentration dependence of the uptake of GHB and D-lactate in Caco-2 cells was determined over a range of concentrations (0.01–80 mM for GHB and 0.05–30 mM for D-lactate) with uptake determined at an extracellular pH of 6.0. The uptake of GHB and D-lactate was described by a Michaelis-Menten equation with passive diffusion (eq. 2) (GHB: $K_m = 17.6 \pm 10.5$ mM, $V_{max} = 17.3 \pm 11.7$ nmol/min/mg, and $P = 0.38 \pm 0.15$ μ l/min/mg; and D-lactate: $K_m = 6.0 \pm 2.9$ mM, $V_{max} = 35.0 \pm 18.4$ nmol/min/mg, and $P = 1.3 \pm 0.6$ μ l/min/mg) (Fig. 3). Eadie-Hofstee plots of GHB and D-lactate uptake suggested the involvement of two transport processes (data not shown); however, the data were not well described by eq. 3, suggesting that two saturable transport processes may not be involved in uptake or that the concentration range assessed was not sufficient to distinguish two transporters.

Influence of MCT Inhibitors. The effect of inhibitors on the transport of GHB and D-lactate was assessed by the incubation of [3 H]GHB or [3 H]D-lactate in the presence or absence of D-lactate, GHB, CHC, or TEA at pH 6.0 (Fig. 4). The uptake of GHB and D-lactate in Caco-2 cells was significantly inhibited in the presence of the MCT substrates and CHC but was not affected by the presence of TEA. These studies indicate the role of MCTs in the uptake of GHB;

FIG. 3. Concentration-dependent uptake of GHB (A) and D-lactate (B) in Caco-2 cells. Experiments were performed at pH 6.0 with uptake determined at 5 min. Data are expressed as mean $\pm$ S.E.M. of two independent experiments performed in triplicate. The line represents the model prediction.

FIG. 4. Effects of MCT inhibitors on GHB (0.1 mM) (A) and D-lactate (0.1 mM) (B) uptake in Caco-2 cells. Experiments were performed at pH 6.0 with D-lactate (5 mM), GHB (0.5 mM), CHC (2 mM), or TEA (5 mM). Results are presented as mean $\pm$ S.E.M. of three independent experiments performed in triplicate. *, $P < 0.05$ compared with control.

however, the specific isoform(s) involved in transport remains to be determined because CHC is a nonspecific MCT inhibitor (Morris and Felmler, 2008).

Directional Flux Studies. Studies were conducted to investigate the directional flux of GHB and D-lactate at different pH values and in the presence of the MCT inhibitor CHC. The AP-to-BL and BL-to-AP transport of both GHB and D-lactate demonstrated no statistically significant difference when pH 7.4 buffers were added to both chambers (Fig. 5). However, the AP-to-BL transport of both GHB and D-lactate was higher than the BL-to-AP transport in the presence of a pH gradient (AP, pH 6.0; BL, pH 7.4) with GHB demonstrating a significantly higher flux in the AP-to-BL direction at 30 and 60 min (Fig. 6, A and B). Under these same pH conditions, there was no difference in the AP-to-BL and BL-to-AP transport for both GHB and D-lactate in the presence of the MCT inhibitor CHC (Fig. 6, C and D). These results suggest that GHB flux is mediated by an apically localized proton-coupled transporter, which is consistent with literature reports regarding the localization of MCT1 in Caco-2 cells (Buyse et al., 2002) and human intestine (Gill et al., 2005).

Discussion

GHB, a drug of abuse, is a four-carbon monocarboxylic acid, which is an endogenous metabolite of GABA. The observed nonlinearity of GHB pharmacokinetics is a result of capacity-limited absorption (Arena and Fung, 1980), metabolism (Lettieri and Fung, 1979; Palatini et al., 1993), and renal clearance (Morris et al., 2005). Previous studies have demonstrated the involvement of MCTs in the saturable renal reabsorption of GHB, contributing to the observed nonlinear renal clearance (Morris et al., 2005; Wang et al., 2008). The present study represents the first investigation of the role of MCTs in the intestinal absorption of GHB. We

FIG. 5. Bidirectional transport of [³H]GHB (A) and [³H]D-lactate (B) across Caco-2 cell monolayers in the absence of a pH gradient. □, permeability from AP to BL; ■, permeability from BL to AP. Data are presented as mean ± S.E.M. of three independent experiments performed in triplicate.

have demonstrated that GHB and D-lactate are taken up into Caco-2 cells in a concentration- and proton gradient-dependent manner, indicating the involvement of MCTs. Their uptake and directional flux were also inhibited by the known MCT inhibitor CHC, as well as the MCT substrates D-lactate and GHB.

In agreement with previous studies, mRNA expression of the MCT isoforms 1, 3, and 4 were observed in Caco-2 cells in the present study (Hadjiagapiou et al., 2000). Our results also indicated, for the first time, mRNA expression of MCT2. The presence of mRNA for MCTs in brush-border or basolateral membrane vesicles from human intestine has been reported previously (Hadjiagapiou et al., 2000; Gill et al., 2005; Englund et al., 2006), with the expression of MCT1 found to be high relative to that of other isoforms in the human intestine (Cundy, 2005). MCT1 localization has been reported to be apical in Caco-2 cells (Buyse et al., 2002) and the localization of additional isoforms of MCTs in Caco-2 cells has not been reported.

GHB and D-lactate transport was demonstrated to occur in a concentration- and proton gradient-dependent manner, suggesting the involvement of MCTs. We observed 3.6- and 3.0-fold increases in uptake when the extracellular pH was decreased from 7.5 to 5.5, which is consistent with the proton-coupled requirement for transport by MCT1-4. In contrast, uptake of GHB and D-lactate occurred in a sodium-independent manner, suggesting that the SMCTs, which are expressed in the colon (Ganapathy et al., 2008), are not involved in uptake in Caco-2 cells. These results are consistent with previous studies in Caco-2 cells examining the transport of butyrate (Hadjiagapiou et al., 2000; Stein et al., 2000) and *p*-coumaric acid (Konishi et al., 2003). Previous studies have indicated that SMCT1 (SLC5A8) is localized on the luminal membrane in the large intestine and contributes to the uptake of monocarboxylates such as L-lactate, pyruvate, and butyrate (Takebe et al., 2005). However, expression of SMCTs is probably absent in Caco-2 cells as these transporters are silenced in colon cancer (Ganapathy et al., 2008). GHB exhibits sodium-dependent transport in a rat thyroid cell line (Cui and Morris, 2009), suggesting that it is a substrate for SMCTs. Although SMCT1 may play a role in the transport of GHB and D-lactate in normal intestinal tissue (Ganapathy et al., 2008), its expression and activity in human tissue are still controversial.

The involvement of MCTs in the uptake of GHB and D-lactate was further confirmed by the significant decrease in uptake after coincubation with a known MCT inhibitor CHC and MCT substrates. These results are consistent with the observed inhibition of MCT-mediated

FIG. 6. Bidirectional transport of [³H]GHB (A) and [³H]D-lactate (B) across Caco-2 cell monolayers in the presence of a pH gradient (apical pH 6.0; basolateral pH 7.4). Bidirectional transport of [³H]GHB (C) and [³H]D-lactate (D) across Caco-2 cell monolayers in the presence of a pH gradient and 2 mM CHC. □, permeability from AP to BL; ■, permeability from BL to AP. Data are presented as mean ± S.E.M. of three independent experiments performed in triplicate except for A, which is two independent experiments performed in triplicate.

transport of butyrate in Caco-2 cells (Hadjiagapiou et al., 2000) and GHB and D/L-lactate in human kidney HK-2 cells (Wang et al., 2007) and rat kidney membrane vesicles (Wang et al., 2006a). However, CHC is a nonspecific inhibitor of MCT1, MCT2, and MCT4 and therefore does not identify the specific MCT isoforms involved in GHB and D-lactate uptake. MCT3-mediated uptake is not inhibited by CHC (Morris and Felmlee, 2008), and it may contribute to residual uptake observed in the presence of CHC. Silencing RNA studies have previously been used to elucidate the involvement of specific MCTs in HK-2 cells and Caco-2 cells. GHB uptake was inhibited 70% in the presence of siRNA for MCT1, whereas uptake was decreased 17% with siRNA for MCT2 in HK-2 cells (Wang et al., 2007), suggesting that MCT1 plays a major role in GHB transport. Further studies in human breast cancer MDA-MB231 cells demonstrated that GHB is a substrate for both MCT2 and MCT4 (Wang and Morris, 2007). Butyrate uptake in Caco-2 cells was significantly inhibited by siRNA for MCT1 (50% inhibition); however, the contribution of other MCT isoforms was not assessed (Lecona et al., 2008). Transient transfection of antisense MCT1 resulted in significant inhibition of butyrate transport, suggesting that MCT1 plays a major role in MCT-mediated transport in Caco-2 cells (Hadjiagapiou et al., 2000).

Although the expression of multiple MCTs was observed in Caco-2 cells, the transport of GHB and D-lactate were best described by a single Michaelis-Menten equation with a diffusional clearance term (eq. 2). Previous studies conducted in our laboratory showed similar kinetic profiles for GHB in HK-2 cells (Wang et al., 2007), rat kidney membrane vesicles, and MCT1-transfected MDA-MB231 cells (Wang et al., 2006a). Uptake studies conducted with D- and L-lactate were best fit to a single Michaelis-Menten equation in HK-2 cells (Wang et al., 2006b, 2007). These differences are also consistent with studies assessing butyrate uptake in HK-2 (Wang et al., 2006b) and Caco-2 (Hadjiagapiou et al., 2000; Lecona et al., 2008) cells. Eadie-Hofstee plots suggested that two transport processes were involved in the uptake of GHB and D-lactate; however, the data were not well described by eq. 3. On the other hand, the data were well described by the inclusion of a diffusional clearance term. The inclusion of a diffusional clearance term in the present study may be a reflection that the concentration range used was not sufficiently wide enough to characterize lower affinity MCT isoforms. MCT1 is probably present in the highest amounts, and uptake may be predominantly due to MCT1. In addition, even though the data are best fit to a single Michaelis-Menten equation, multiple MCTs may be involved in the transport of GHB and D-lactate but have similar affinities and therefore could not be distinguished in concentration-dependent uptake studies. Further studies are needed to elucidate the specific MCTs that are involved in GHB and D-lactate uptake in Caco-2 cells. The K_m value for GHB (17.6 mM) obtained in the current study is higher than that observed in HK-2 (2.07 mM) (Wang et al., 2007) and rat MCT1-transfected MDA-MB231 cells (4.6 mM) (Wang et al., 2006a), which may suggest the involvement of other MCTs besides MCT1. In contrast, the K_m for D-lactate (6.0 mM) is lower than that observed in HK-2 cells (26.5 mM); this may be a result of the different kinetic models used to describe the data in these two cell lines, because the model in HK-2 cells did not include a passive diffusion component.

MCT1 has been shown to be localized on the brush-border membranes of cells in the human small intestine and brush border and/or basolateral membranes of cells in the colon (Tamai et al., 1999; Gill et al., 2005; Iwanaga et al., 2006). In Caco-2 cells, MCT1 localization at the brush border (apical) membrane has been demonstrated (Cuff et al., 2002); however, basolateral membrane expression was not assessed. Directional transport with a greater transport rate in the AP-to-BL direction was observed in the presence of a physiological pH

gradient (pH 6.0 on the apical side and pH 7.4 on the basal side), which is consistent with absorption of GHB across the intestinal epithelium. Directional flux disappeared in the absence of a pH gradient or in the presence of the MCT inhibitor CHC, indicating that MCTs play a role in GHB and D-lactate flux. MCT1 has been demonstrated to be important in the intestinal uptake of substrates (Li et al., 1999; Cundy et al., 2004). In fact, gabapentin has been formulated as a prodrug for MCT1 to increase its bioavailability (Cundy et al., 2004). Substrates of MCT1 demonstrate nonlinear absorption and bioavailability consistent with facilitated uptake (Arena and Fung, 1980; Cundy et al., 2004). Although MCT1 expression on the basolateral membrane of Caco-2 cells has not been reported, there are literature reports indicating that MCT1 is expressed on the basolateral membrane of intestinal cells, where it appears to function as an efflux transporter (Huang et al., 2009). Efflux of MCT1 substrates at the basolateral membrane of the intestine would contribute to the observed directional flux from the apical to basolateral membranes, as observed in the present study, and to the overall intestinal absorption of the substrate.

One complicating factor in these studies is the potential metabolism of GHB over the time course of the flux study; however, short incubation times were used to minimize metabolism of GHB. D-Lactate would not be expected to be metabolized to any extent (Ullrich et al., 1982). Similar flux characteristics for these two substrates suggest that MCTs play a role in the transport of GHB and D-lactate across Caco-2 cells. Polarized flux greater in the apical to basolateral direction has also been reported in Caco-2 cells for the MCT substrate *p*-coumaric acid (Konishi et al., 2003).

In summary, the presence of MCT1–4 mRNA in Caco-2 cells was detected. The proton-dependent nature of the uptake and the inhibition by MCT substrates and the classic MCT inhibitor CHC suggest that GHB and D-lactate are transported by MCTs in Caco-2 cells. In the presence of a physiological pH gradient, MCTs contribute to the flux of GHB and D-lactate across human intestinal Caco-2 cells. Our findings in Caco-2 cells are in agreement with previous reports of the MCT-mediated transport of GHB and D-lactate in HK-2 and MDA-MB231 cells (Wang et al., 2006a, 2007; Wang and Morris, 2007). Further research is needed to determine the contribution of each MCT isoform and the potential role of sodium-dependent monocarboxylate transporters in the intestinal transport of monocarboxylates.

Acknowledgments. We acknowledge valuable contributions from Drs. Qi Wang and Xiaodong Wang.

References

- Arena C and Fung HL (1980) Absorption of sodium γ -hydroxybutyrate and its prodrug γ -butyrolactone: relationship between in vitro transport and in vivo absorption. *J Pharm Sci* **69**:356–358.
- Bonen A, Heynen M, and Hattat H (2006) Distribution of monocarboxylate transporters MCT1–MCT8 in rat tissues and human skeletal muscle. *Appl Physiol Nutr Metab* **31**:31–39.
- Buyse M, Sitaraman SV, Liu X, Bado A, and Merlin D (2002) Luminal leptin enhances CD147/MCT-1-mediated uptake of butyrate in the human intestinal cell line Caco2-BBE. *J Biol Chem* **277**:28182–28190.
- Cuff MA, Lambert DW, and Shirazi-Beechey SP (2002) Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. *J Physiol* **539**:361–371.
- Cui D and Morris ME (2009) The drug of abuse γ -hydroxybutyrate is a substrate for sodium-coupled monocarboxylate transporter (SMCT) 1 (SLC5A8): characterization of SMCT-mediated uptake and inhibition. *Drug Metab Dispos* **37**:1404–1410.
- Cundy KC (2005) *Novel Uses of Drug Transporters for Drug Delivery: A Case Study with Gabapentin*. Xenopharm, Inc., Santa Clara, CA.
- Cundy KC, Annamalai T, Bu L, De Vera J, Estrela J, Luo W, Shirsat P, Torneros A, Yao F, Zou J, Barrett RW, and Gallop MA (2004) XP13512 [($\pm$)-1-((α -isobutanoyloxyethoxy)carbonyl)aminomethyl]-1-cyclohexane acetic acid], a novel gabapentin prodrug: II. Improved oral bioavailability, dose proportionality, and colonic absorption compared with gabapentin in rats and monkeys. *J Pharmacol Exp Ther* **311**:324–333.
- Englund G, Rorsman F, Rönblom A, Karlsson U, Lazorova L, Gråsjö J, Kindmark A, and Artursson P (2006) Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur J Pharm Sci* **29**:269–277.

- Ganapathy V, Thangaraju M, Gopal E, Martin PM, Itagaki S, Miyauchi S, and Prasad PD (2008) Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. *AAPS J* **10**:193–199.
- Gill RK, Sakkena S, Alrefai WA, Sarwar Z, Goldstein JL, Carroll RE, Ramaswamy K, and Dudeja PK (2005) Expression and membrane localization of MCT isoforms along the length of the human intestine. *Am J Physiol Cell Physiol* **289**:C846–C852.
- Hadjigapigou C, Schmidt L, Dudeja PK, Layden TJ, and Ramaswamy K (2000) Mechanism(s) of butyrate transport in Caco-2 cells: role of monocarboxylate transporter 1. *Am J Physiol Gastrointest Liver Physiol* **279**:G775–G780.
- Halestrap AP and Meredith D (2004) The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflugers Arch* **447**:619–628.
- Halestrap AP and Price NT (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J* **343** (Pt 2):281–299.
- Huang W, Lee SL, and Yu LX (2009) Mechanistic approaches to predicting oral drug absorption. *AAPS J* **11**:217–224.
- Iwanaga T, Takebe K, Kato I, Karaki S, and Kuwahara A (2006) Cellular expression of monocarboxylate transporters (MCT) in the digestive tract of the mouse, rat, and humans, with special reference to slc5a8. *Biomed Res* **27**:243–254.
- Juel C and Halestrap AP (1999) Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter. *J Physiol* **517** (Pt 3):633–642.
- Konishi Y, Kobayashi S, and Shimizu M (2003) Transepithelial transport of *p*-coumaric acid and gallic acid in Caco-2 cell monolayers. *Biosci Biotechnol Biochem* **67**:2317–2324.
- Lecona E, Olmo N, Turnay J, Santiago-Gómez A, López de Silanes I, Gorospe M, and Lizarbe MA (2008) Kinetic analysis of butyrate transport in human colon adenocarcinoma cells reveals two different carrier-mediated mechanisms. *Biochem J* **409**:311–320.
- Lettieri JT and Fung HL (1979) Dose-dependent pharmacokinetics and hypnotic effects of sodium γ -hydroxybutyrate in the rat. *J Pharmacol Exp Ther* **208**:7–11.
- Li YH, Ito K, Tsuda Y, Kohda R, Yamada H, and Itoh T (1999) Mechanism of intestinal absorption of an orally active β -lactam prodrug: uptake and transport of carindacillin in Caco-2 cells. *J Pharmacol Exp Ther* **290**:958–964.
- Lin RY, Vera JC, Chaganti RS, and Golde DW (1998) Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. *J Biol Chem* **273**:28959–28965.
- Manning Fox JE, Meredith D, and Halestrap AP (2000) Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. *J Physiol* **529** (Pt 2):285–293.
- Morris ME and Felmler MA (2008) Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse γ -hydroxybutyric acid. *AAPS J* **10**:311–321.
- Morris ME, Hu K, and Wang Q (2005) Renal clearance of γ -hydroxybutyric acid in rats: increasing renal elimination as a detoxification strategy. *J Pharmacol Exp Ther* **313**:1194–1202.
- Murakami Y, Kohyama N, Kobayashi Y, Ohbayashi M, Ohtani H, Sawada Y, and Yamamoto T (2005) Functional characterization of human monocarboxylate transporter 6 (SLC16A5). *Drug Metab Dispos* **33**:1845–1851.
- Palatini P, Tedeschi L, Frison G, Padriani R, Zordan R, Orlando R, Gallimberti L, Gessa GL, and Ferrara SD (1993) Dose-dependent absorption and elimination of γ -hydroxybutyric acid in healthy volunteers. *Eur J Clin Pharmacol* **45**:353–356.
- Philp NJ, Wang D, Yoon H, and Hjelmeland LM (2003) Polarized expression of monocarboxylate transporters in human retinal pigment epithelium and ARPE-19 cells. *Invest Ophthalmol Vis Sci* **44**:1716–1721.
- Poldrugo F and Addolorato G (1999) The role of γ -hydroxybutyric acid in the treatment of alcoholism: from animal to clinical studies. *Alcohol Alcohol* **34**:15–24.
- Scharf MB, Lai AA, Branigan B, Stover R, and Berkowitz DB (1998) Pharmacokinetics of γ -hydroxybutyrate (GHB) in narcoleptic patients. *Sleep* **21**:507–514.
- Shah P, Jogani V, Bagchi T, and Misra A (2006) Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. *Biotechnol Prog* **22**:186–198.
- Stein J, Zores M, and Schröder O (2000) Short-chain fatty acid (SCFA) uptake into Caco-2 cells by a pH-dependent and carrier mediated transport mechanism. *Eur J Nutr* **39**:121–125.
- Takebe K, Nio J, Morimatsu M, Karaki S, Kuwahara A, Kato I, and Iwanaga T (2005) Histochemical demonstration of a Na^+ -coupled transporter for short-chain fatty acids (slc5a8) in the intestine and kidney of the mouse. *Biomed Res* **26**:213–221.
- Tamai I, Sai Y, Ono A, Kido Y, Yabuuchi H, Takahara H, Satoh E, Ogihara T, Amano O, Izeki S, and Tsuji A (1999) Immunohistochemical and functional characterization of pH-dependent intestinal absorption of weak organic acids by the monocarboxylic acid transporter MCT1. *J Pharm Pharmacol* **51**:1113–1121.
- Ullrich KJ, Rumrich G, and Klöss S (1982) Reabsorption of monocarboxylic acids in the proximal tubule of the rat kidney. I. Transport kinetics of D-lactate, Na^+ -dependence, pH-dependence and effect of inhibitors. *Pflugers Arch* **395**:212–219.
- Wang Q, Darling IM, and Morris ME (2006a) Transport of γ -hydroxybutyrate in rat kidney membrane vesicles: role of monocarboxylate transporters. *J Pharmacol Exp Ther* **318**:751–761.
- Wang Q, Lu Y, and Morris ME (2007) Monocarboxylate transporter (MCT) mediates the transport of γ -hydroxybutyrate in human kidney HK-2 cells. *Pharm Res* **24**:1067–1078.
- Wang Q, Lu Y, Yuan M, Darling IM, Repasky EA, and Morris ME (2006b) Characterization of monocarboxylate transport in human kidney HK-2 cells. *Mol Pharm* **3**:675–685.
- Wang Q and Morris ME (2007) The role of monocarboxylate transporters 2 and 4 in the transport of γ -hydroxybutyric acid in mammalian cells. *Drug Metab Dispos* **35**:1393–1399.
- Wang Q, Wang X, and Morris ME (2008) Effects of L-lactate and D-mannitol on γ -hydroxybutyrate toxicokinetics and toxicodynamics in rats. *Drug Metab Dispos* **36**:2244–2251.
- Zhang S and Morris ME (2003) Effect of the flavonoids biochanin A and silymarin on the P-glycoprotein-mediated transport of digoxin and vinblastine in human intestinal Caco-2 cells. *Pharm Res* **20**:1184–1191.
- Zhu S, Goldschmidt-Clermont PJ, and Dong C (2005) Inactivation of monocarboxylate transporter MCT3 by DNA methylation in atherosclerosis. *Circulation* **112**:1353–1361.

Address correspondence to: Dr. Marilyn E. Morris, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, Buffalo, NY 14260. E-mail: memorris@buffalo.edu