Transport of γ-Hydroxybutyrate in Rat Kidney Membrane Vesicles: Role of Monocarboxylate Transporters

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Received April 9, 2006; accepted May 16, 2006

ABSTRACT

Intoxication with γ-hydroxybutyrate (GHB) is associated with coma, seizure, and death; treatment of overdoses is symptomatic. Previous studies in our laboratory have demonstrated that L-lactate and pyruvate treatment can increase the renal clearance of GHB and increase its elimination in rats, suggesting that GHB may undergo renal reabsorption mediated by monocarboxylic acid transporters (MCTs). The goals of this study were to characterize the renal transport of GHB in rats and to determine the role of MCT in its renal transport. Brush-border membrane (BBM) and basolateral membrane (BLM) vesicles were isolated from rat kidney cortex, and the uptake of L-lactate and GHB was characterized. L-Lactate and GHB undergo both pH- and sodium-dependent transport in BBM vesicles and pH-dependent transport in BLM vesicles. A simple Michaelis-Menten equation best described the pH-dependent uptake of GHB in BBM (Km, 8.0 ± 1.8 mM; Vmax, 838 ± 45 pmol/mg/s) and in BLM vesicles (Km, 10.5 ± 2.6 mM; Vmax, 806 ± 253 pmol/mg/s). mRNA of MCT1 and MCT2 was determined in rat kidney cortex using reverse transcriptase-polymerase chain reaction; using Western blot, the protein expression of MCT1 was present mainly in BLM vesicles, with weak expression in BBM vesicles, whereas that of MCT2 was exclusively in BLM vesicles. Studies with rat MCT1 gene-transfected MDA-MB231 cells demonstrated that GHB was a substrate of MCT1. The data suggest that rat MCT1 may represent an important transporter for GHB in renal tubule cells. This investigation provides evidence for the importance of MCTs in the reabsorption of the monocarboxylic acids L-lactate and GHB in the kidney.

γ-Hydroxybutyrate (GHB) is a naturally occurring short-chain fatty acid, formed from γ-aminobutyric acid (Maitre, 1997), that is present in the mammalian brain, heart, liver, and kidney (Roth and Giarman, 1970; Maitre, 1997). GHB has been used in Europe, beginning in the late 1970s, for treatment of the sleep disorder narcolepsy (Mamelak et al., 1986), and in the 1980s, for treatment of alcohol and heroin dependence (Gallimberti et al., 2000). In 1990, GHB was marketed as a dietary supplement in the United States, but was rapidly banned by the Food and Drug Administration from over-the-counter sales due to wide-spread reports of abuse (Galloway et al., 1997). Deaths resulted from the use of GHB by body builders as a steroid alternative (Okun et al., 2001) and from its use as a recreational drug, due to its euphoric effects, at nightclubs and rave parties (Wong et al., 2004). GHB has been used in drug-facilitated sexual assaults (date rape) due to its hypnotic/amnesic effects. In 2002, GHB (Xyrem) was approved by the Food and Drug Administration as an orphan medication for treating a small population of patients with narcolepsy who experience episodes of cataplexy but was classified as a Schedule III drug, and its distribution was tightly restricted.

The adverse effects of GHB following intoxication result primarily from central nervous system and respiratory depression and, to a lesser extent, from effects on the cardiovascular and gastrointestinal systems (Mason and Kerns, 2002). GHB and its precursors (γ-butyrolactone and 1,4-butanediol) are especially toxic when mixed with alcohol and/or other recreational drugs to increase their euphoric effects. These combinations have led to significant morbidity and mortality (Okun et al., 2001). However, the treatment of GHB overdose is very limited, consisting mainly of supportive care. There have been no specific antidotes reported for clinical use, although flumazenil, naloxone, and phystostigmine have been tried (Mason and Kerns, 2002).

ABBREVIATIONS: GHB, γ-hydroxybutyrate; MCT, monocarboxylate transporter; BBM, brush-border membrane; BLM, basolateral membrane; AA, acetooacetate; BHB, β-hydroxybutyrate; BTD, 1,4-butanediol; CHC, α-cyano-4-hydroxycinnamate; DIDS, 4,4′-disothiocyanatostilbene-2,2′-disulfonic acid disodium salt; TEA, tetraethylammonium; MES, 2-(N-morpholino)ethanesulfonic acid; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; RT, reverse transcriptase; PCR, polymerase chain reaction; ANOVA, analysis of variance; bp, base pair(s); GBL, γ-butyrolactone.
The pharmacokinetics of GHB has been found to be non-linear in rats (Lettieri and Fung, 1979) and humans (Ferrara et al., 1992; Palatini et al., 1993), with decreasing total clearance with increasing dose. The mechanisms underlying the non-linear pharmacokinetics of GHB may include capacity-limited metabolism (Lettieri and Fung, 1979; Ferrara et al., 1992; Palatini et al., 1993) and/or absorption (Arena and Fung, 1980). Following the administration of low doses, the urinary recovery of GHB is 5 to 7% in rats (Lettieri and Fung, 1976) and <1% in humans (Ferrara et al., 1992). However, the renal clearance of GHB may be more important following overdoses since it has been reported that intoxicated patients who ingested GHB, or the precursors of GHB, γ-butyrolactone and 1,4-butanediol, had high concentrations of GHB in urine samples (Zvosec et al., 2001; Sporer et al., 2003). In a recent study, we found that the renal clearance of GHB also contributed to the non-linear pharmacokinetics of GHB (Morris et al., 2005). The renal clearance of GHB is significantly increased at higher GHB plasma concentrations and plays a more important role in the overall elimination of GHB following overdoses. The non-linear renal clearance of GHB may be due to a carrier-mediated renal reabsorption by monocarboxylic acid transporters (MCTs) since we observed that the MCT substrates, L-lactate and pyruvate, could inhibit the active reabsorption and increase the renal clearance of GHB (Morris et al., 2005). Among the 14 members (MCT1–14) in the MCT family, only MCT1–4 have been shown to be proton-coupled monocarboxylate transporters (Halestrap and Price, 1999; Halestrap and Meredith, 2004), with similar, but not identical, substrates. Among these four MCTs, MCT1, the first member identified, is ubiquitously distributed in the body and is present in red blood cells, intestine, colon, muscle, heart, brain, and kidney (Halestrap and Meredith, 2004). MCT2 shares 60% identity with MCT1 but with much limited tissue distribution, including liver, brain, and kidney. The renal transport mechanisms of GHB have not been well studied, and it is not known whether it represents a MCT substrate in the kidney. The objectives of this study were to investigate transport driving forces of GHB in brush-border membrane (BBM) and basolateral membrane (BLM) vesicles isolated from rat kidney cortex, to study the transport kinetics of GHB in BBM and BLM vesicles, to evaluate expression of rat MCT1 and MCT2 in BBM and BLM, to investigate the role of rat MCT1 as a GHB transporter in rat kidney cortex, and to characterize the transport and inhibition of GHB in rat MCT1 gene-transfected cells. The transport characteristics of GHB were compared with that of L-lactate, a known MCT substrate.

Materials and Methods

Chemicals. GHB, L-lactate, D-lactate, pyruvate, acetacetate (AA), β-hydroxybutyrate (BHB), 1,4-butanediol (BTD), o-cyano-4-hydroxycinnamate (CHC), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt (DIDS), phloretin, tetraethylammonium (TEA), HEPES, and MES were purchased from Sigma-Aldrich (St. Louis, MO). D-[2-3H]Lactate (20 Ci/mmol) and L-[14C (U)]-lactate (56 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). GHB (35–50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA).

Rat Kidney Membrane Vesicle Preparation. Membrane vesicles were prepared as described previously (Darling et al., 1994). All the preparation steps were conducted on ice. In brief, kidney cortex tissues from three Sprague-Dawley rats were homogenized in homogenizing buffer (250 mM sucrose, 10 mM triethanolamine-HCl, pH 7.5). BBM and BLM vesicles were separated by self-oriented Percoll (Sigma-Aldrich) density gradient centrifugation at 4°C. The BBM fraction was further purified with MgCl2 precipitation. The final BBM and BLM preparations were resuspended in vesicle buffer (300 mM mannitol, 20 mM HEPES, pH 7.5). The purity of BBM vesicles prepared was determined by the enrichment of the enzymes alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT), and the purity of BLM vesicles was determined by the enrichment of the enzyme Na+/K+-ATPase. Alkaline phosphatase activity was determined using a commercially available kit (Sigma Diagnostics, kit 245). γ-Glutamyl transpeptidase activity was assayed as described previously (Darling et al., 1994). Na+/K+-ATPase was determined by the method of Jegensen and Skou as described previously (Darling et al., 1994). Protein content of the membrane vesicles was determined by a reagent (Bio-Rad, Hercules, CA) using γ-immunoglobulin as the protein standard.

Uptake Studies Using Rat Kidney Cortex Membrane Vesicles. The transport of GHB and L-lactate were measured in membrane vesicles using a rapid filtration method as described previously (Darling et al., 1994). The uptake reaction was initiated by adding vesicles (10–12 mg/ml) to an uptake medium containing radiolabeled [3H]GHB or L-[14C]lactate in a 1:10 dilution (final protein concentration was 0.1–0.12 mg/ml). Uptake was stopped by adding 1 ml of ice-cold stop buffer (300 mM mannitol and 20 mM HEPES, pH 7.5). The stop-reaction mixture was transferred immediately to a membrane filter (Type HA, 0.45 μm; Millipore Corp., Bedford, MA) and washed with 5 ml of ice-cold buffer with vacuum filtration. The filters were preincubated overnight in a vesicle buffer containing 100 mM GHB or L-lactate to minimize any nonspecific binding of the substrates to the filter itself. The uptake medium was maintained in a water-bath (Precision Scientific, Chicago, IL) at 25°C prior to and throughout the uptake procedure. The binding of substrates to the filters was determined by filtering uptake medium without vesicles, and the values were deducted in the calculation of uptake values. The filters were dissolved in 1 ml of 2-methoxyethanol in 6-ml polystyrene scintillation vials (Beckman, Sommerville, NJ), and 3 ml of biodegradable scintillation fluid was added for counting by liquid scintillation spectrometry (Tri-Carb 1900CA; Packard Instrument Co., Downers Grove, IL).

To evaluate the potential driving force for the uptake of GHB or L-lactate by BBM and BLM vesicles, the uptake of [3H]GHB or L-[14C]lactate was determined using pH, Na+, or bicarbonate gradients. Uptake buffers of various pH values and lacking Na+ (300 mM mannitol, 20 mM MES or HEPES, 40 μM GHB) were used to examine the time course of pH-dependent uptake of GHB or L-lactate by BBM and BLM vesicles. An uptake buffer of pH 7.5 containing 100 mM NaCl, 100 mM mannitol, and 20 mM HEPES, was used to examine the time course of sodium-dependent uptake of [3H]GHB or L-[14C]lactate by BBM and BLM vesicles. To evaluate a possible anion exchange mechanism for GHB uptake by BBM and BLM vesicles, the vesicles were preloaded with or without 100 mM potassium bicarbonate, and the uptake of GHB was examined. From the time course studies, it was found that a 2-s uptake value could be used for the determination of the linear uptake rate for the Na+-dependent uptake of GHB and a 10-s uptake value for the pH-dependent uptake of GHB.

MCT substrates, L-lactate, and pyruvate were used to inhibit the uptake of GHB by BBM and BLM vesicles. Some structurally similar compounds, such as β-hydroxybutyrate and 1,4-butanediol, were also used as possible inhibitors. Transporter inhibitors, including MCT inhibitors (CHC and phloretin), Oat inhibitors (salicylic acid and ketoprofen), Oatp inhibitor (probenevid), and anion exchanger inhibitors (SITS) were used to inhibit GHB uptake by BBM and BLM vesicles. The effects of these compounds on the uptake of [3H]GHB or L-[14C]lactate by BBM and BLM were examined under specific con-
Cell Culture and Cellular Uptake Study. Rat MCT1 gene-transfected MDA-MB231 cells and mock cells were kindly provided by Professors I. Tamai and A. Tsuji (Kanazawa University, Kanazawa, Japan), and cultured as described previously (Tamai et al., 1999). In brief, cells were cultured in T 75-cm² flasks in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum, 1 mg/ml genetin (G418; Invitrogen), 100 units of penicillin, and 100 µg/ml streptomycin added, and the fresh media added every 2 to 3 days. Cells were maintained at 37°C in 5% CO₂/95% air environment. Cells were seeded in 35-mm-diameter plastic culture dishes 2 to 3 days before uptake studies. For the uptake study, the cells were removed from cell monolayers, and cells were washed three times with uptake buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4). One milliliter of uptake buffer containing [³H]GHB or [³H]lactate was added to the dishes. d-Lactate, and not l-lactate, was used in the cell studies due to the rapid metabolism of l-lactate in cell studies. For the time course study, the cells were incubated at room temperature for 0.5, 1, 5, 10, 30, and 60 min. For the pH-dependent study, the cells were incubated with uptake buffer with different pH values (pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0), whereas for the sodium-dependent study, the cells were incubated with sodium buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5). Inhibitors used in the cellular uptake studies included CHC, DIDS, TEA, phloretin, and b-lactate. The uptake was stopped by aspirating the buffer and washing three times with ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5). Inhibitors used in the cellular uptake studies included CHC, DIDS, TEA, phloretin, and b-lactate. The uptake was stopped by aspirating the buffer and washing three times with ice-cold stop buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5). The cells were lysed and collected in 1 ml of lysis buffer containing 0.3 M NaOH and 1% SDS. Radioactivity was determined by liquid scintillation spectroscopy. Protein concentrations were determined by the bicinchoninic acid protein assay with bovine serum albumin as the protein standard. The results were normalized for the protein content of the cell lysate, and the accumulation was expressed as picomoles or nanomoles per milligram of protein per minute.

Reverse Transcripase-PCR. Total RNA was isolated from rat kidney cortex with the SV total RNA isolation system (Promega, Madison, WI). First strand cDNA was synthesized from total RNA by reverse transcription (RT) using an oligo(dT) primer with Brilliant Two-Step QRT-PCR core reagent kit (Cedar Creek, TX). PCR was performed using an Eppendorf Mastercycler gradient PCR system (Eppendorf Scientific Inc., Westbury, NY). The primers specific to rat MCT1 and MCT2 were designed using Primer Express software and checked in the National Center for Biotechnology Information database for sequence identity (MCT1, forward, 5'-ACCCGAGACATCCGAAACC-3'; reverse, 5'-AATTGTCCACTGTCTGCACGG-3'; MCT2, forward, 5'-CCTCTTT-AACCGAGACATCCGAAACC-3'; reverse, 5'-TTATCGGAGAATTACCCGAG-3'). The PCR reaction mixtures contained 200 µl of each dNTP, 0.2 µM of each forward and reverse primer, and 0.5 U reaction TaqDNA polymerase 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 0.5 min, 60°C for 0.5 min, and 72°C for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel and then stained by ethidium bromide and visualized under UV light.

Western Blot. The expression of rat MCT1 and MCT2 in MDA-MB231 cells and rat kidney cortex were examined by Western blotting. MDA-MB231 cell total lysates were prepared as follows: the cells were collected and centrifuged at 13,000 g for 5 min, the cell pellet was lysed with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-Cl, pH 7.4, 5 mM dithiothreitol, 100 µM phenylmethylsulfonyl fluoride in isopropanol, and 5 mM e-amino-caproic acid for 30 min and then centrifuged at 16,000 g for 15 min at 4°C. BBM and BLM vesicles were prepared as described previously. Lysates were separated on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and then transferred to nitrocellulose membrane. Nonspecific protein binding sites were blocked by 5% (w/v) fat-free dry milk (Bio-Rad) in Tris-buffered saline containing 0.1% (v/v) Tween 20 at 4°C overnight. The nitrocellulose membrane was then incubated with the primary antibodies for MCT1 and MCT2 (2 µg/ml) (Chemicon, Temecula, CA) overnight. After incubating with secondary antibody (Chemicon), immunoblots were developed with the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Data Analysis. The data are presented as mean ± S.D. Statistical significance among means of more than two groups was determined by one-way analysis of variance (ANOVA) followed by a Dunnett’s post hoc test. A difference with a p value of 0.05 or less was considered as statistically significant. Data analysis was performed using GraphPad Prism (GraphPad Inc., San Diego CA). The transport kinetic parameters, Michaelis-Menten constant (Kₘ), and maximum uptake rate (Vₘₐₓ), were determined by fitting the data using weighted nonlinear regression analysis (Winlino 2.1; Pharsight Corp., Cary, NC) and the following equations:

\[ v = \frac{V_{\text{max}} \times C}{K_m + C} \]  (1)

\[ v = \frac{V_{\text{max}} \times C}{K_m + P \times C} \]  (2)

\[ v = P \times C \]  (3)

where v is the uptake rate of GHB, C is the concentration of GHB, and P is the nonsaturable uptake parameter. The goodness of fit was determined by the visual examination of fitting curve, the sum of the squared derivatives, the residual plot, and the Akaike Information Criterion. The equation that provided the smallest coefficient of variation percentage and Akaike Information Criterion for the data with the same weighting scheme was used in obtaining Kₘ and Vₘₐₓ parameters for the uptake data.

Results

Membrane Vesicle Characterization

Kidney homogenates and membrane vesicles preparations were assayed for the BLM-specific enzyme Na⁺/K⁺-ATPase and BBM-specific enzymes GGT and ALP to determine the purity of the membrane vesicles preparations. Na⁺/K⁺-ATPase activity was increased in basolateral membrane fractions with a mean enrichment ratio of 6.7-fold (BLM/tissue homogenate), whereas GGT and ALP were enriched in brush-border membrane fractions with mean ratios of 14- and 15-fold, respectively. These enrichment ratios agree well with previous literature values (Darling et al., 1994; Gutierrez et al., 1996).

Rat MCT1 and MCT2 Gene Expression and Protein Expression

Using RT-PCR, we demonstrated the presence of rat MCT1 and MCT2 mRNA in rat kidney cortex homogenates (Fig. 1A). Using Western blot, the cellular membrane localizations of rat MCT1 and MCT2 were analyzed. Protein expression of rat MCT1 was present in BLM, with much lower expression in BBM (Fig. 1B). The protein of rat MCT2 was distributed predominantly in the BLM, with no little expression in BBM (Fig. 1C). We also demonstrated the presence of rat MCT1 protein in rat MCT1 gene-transfected MDA-MB231 cells but not in control MDA-MB231 cells (Fig. 1B).
Studies Examining L-Lactate and GHB Uptake

Driving Forces for the Uptake of L-Lactate in BBM.

In the presence of a sodium gradient, the uptake of [14C]lactate by BBM vesicles displayed an overshoot phenomenon (Fig. 2A), which agreed with previous reports (Barac-Nieto et al., 1980; Nord et al., 1982). We have demonstrated the pH-dependent transport for L-lactate in BBM vesicles (Fig. 2B), which agreed with previous reports (Barac-Nieto et al., 1980; Nord et al., 1982). We have demonstrated the pH-dependent transport for L-lactate in BBM vesicles (Fig. 2B).

Driving Forces for the Uptake of L-Lactate in BLM.

In the presence of an outside-to-inside pH gradient, the time profile of L-lactate uptake by BLM vesicles exhibited an overshoot phenomenon (Fig. 2C), which suggested a transporter-mediated uptake of L-lactate. No sodium-dependent transport of L-lactate in BLM vesicles was observed (data not shown).

Effects of Various Inhibitors on the Uptake of L-Lactate. BBM. Since there were two driving forces for the uptake of L-lactate in BBM vesicles, we studied the effects of various compounds on L-lactate uptake by BBM vesicles in the presence of either a sodium or pH gradient as the driving force. In the presence of a sodium gradient, the uptake of L-lactate by BBM vesicles could only be inhibited by AA (2 mM) (Fig. 2E), but not by BTD (2 mM), BHB (2 mM), and CHC (2 mM) (Fig. 2F). In the presence of a pH gradient, the uptake of L-lactate by BBM vesicles was significantly decreased in the presence of AA (2 mM). The MCT inhibitor, CHC, significantly decreased the uptake of L-lactate in BBM vesicles (Fig. 2D). The GHB precursor, BTD (2 mM), GBL (2 mM), and the GHB isomer, BHB (2 mM), had no effect on L-lactate uptake in BBM vesicles.

BLM. The effect of inhibitors on the uptake of GHB in BLM vesicles was determined at pH 5.5. In the presence of AA (2 mM), BTD (2 mM), GBL (2 mM), and BHB (2 mM), the uptake of L-lactate was significantly decreased (Fig. 2F). The MCT inhibitor, CHC, significantly decreased the uptake of GHB in BLM vesicles (Fig. 2F).

Driving Forces for the Uptake of GHB by BBM.

In the presence of a sodium gradient, the uptake of GHB by BBM vesicles displayed an overshoot phenomenon (Fig. 3A). This overshoot phenomenon was not seen with a potassium gradient (data not shown). Similar to what we observed for L-lactate, an overshoot phenomenon was seen for the uptake of GHB in the presence of a pH gradient (Fig. 3B). The uptake of GHB was not enhanced in the presence of an inside-outside potassium bicarbonate gradient, which suggested that anion exchange was not involved in GHB uptake by BBM vesicles (Fig. 3C).

Concentration-Dependent Uptake of GHB Uptake in BBM. The uptake of GHB in BBM membrane vesicles was decreased with an increase of pH, with the highest uptake of GHB at pH 5.5, and lowest uptake at pH 7.5 (Fig. 4A). From the time course of GHB uptake determined at pH 5.5, a 10-s uptake time was chosen to provide an estimate of the linear uptake rate and was used to determine the concentration-dependent uptake of GHB. The total uptake of GHB could be best fitted with eq. 2, and the uptake determined at pH 7.5 was best fitted with a nonsaturable diffusion equation (eq. 3). The net uptake of GHB by BBM (uptake at pH 5.5 minus uptake at pH 7.5) was best fitted by a single Michaelis-Menten equation (eq. 1) with a \( K_m \) of 8.0 ± 1.8 mM and \( V_{max} \) of 838 ± 48 pmol/mg/s (Table 1). Simultaneously fitting of all three data sets gave similar \( K_m \) and \( V_{max} \) values. A 2-s uptake time, based on the time course studies in the presence of a sodium gradient, was found to represent the linear uptake rate in BBM and was used to determine the concentration-dependent uptake of GHB. The concentration-dependent uptake study in the presence of a sodium gradient, however, did not demonstrate saturation, even with GHB concentrations as high as 50 mM (Fig. 4C).

Effects of Various Inhibitors on the Uptake of GHB in BBM. Since there were two driving forces for the uptake of GHB in BBM vesicles, we studied the effects of various compounds on GHB uptake by BBM vesicles in the presence of either a pH or sodium gradient as the driving force. In the presence of a sodium gradient, the uptake of GHB by BBM vesicles was significantly decreased in the presence of AA (2 mM), D-lactate (2 mM), pyruvate (2 mM), and L-lactate (2 mM). The GHB precursor, BTD (2 mM), and the GHB isomer, BHB (2 mM), had no effect on GHB uptake. The MCT inhibitor, CHC, significantly decreased the uptake of GHB in BBM vesicles (Fig. 4D). In the presence of a sodium gradient, the uptake of GHB by BBM vesicles can be inhibited by monocarboxylates, such as D-lactate (2 mM), pyruvate (2 mM), AA (2 mM), but not by L-lactate (2 mM), and not by its isomer, BHB (2 mM) or its precursor BTD. In addition, the MCT inhibitor CHC (2 mM) had no effect on GHB uptake (Fig. 4E).

Driving Forces for the Uptake of GHB by BLM. In the presence of a sodium gradient, as used in BBM vesicles, no sodium-dependent transport of GHB or L-lactate was demonstrated, as shown in their time profiles (Fig. 5A). Higher concentrations of GHB also showed no sodium-dependent uptake (data not shown). However, in the presence of a pH
gradient, the time course of GHB uptake by BLM vesicles exhibited an overshoot phenomenon (Fig. 5B). Preloading BLM vesicles with bicarbonate had no effect on the uptake of GHB (Fig. 5C), suggesting that an anion exchanger was not involved in GHB transport.

**Concentration-Dependent Uptake of GHB Uptake in BLM.** The uptake of GHB in BLM vesicles decreased with an increase of external buffer pH, with the highest uptake of GHB at pH 5.5 and lowest uptake at pH 7.5 (Fig. 6A). A 10-s uptake time, based on the time course studies at pH 5.5, was found to represent the linear uptake rate in BLM and was used to determine the concentration-dependent uptake of GHB in BLM at pH 5.5. The net uptake of GHB by BLM vesicles was best fitted to a single Michaelis-Menten equation (eq. 1), with a $K_m$ of 10.5 ± 2.6 mM and $V_{max}$ of 806 ± 253 pmol/mg/s (Table 1). Likewise, all three data sets could be fitted simultaneously and gave similar $K_m$ and $V_{max}$ values (Fig. 6B).

**Effects of Various Inhibitors on the Uptake of GHB in BLM.** The effect of inhibitors on the uptake of GHB in BLM vesicles was determined with an external buffer pH of 5.5. In the presence of AA (2 mM), pyruvate (2 mM), D-lactate (2 mM), and L-lactate (10 mM), the uptake of GHB was significantly decreased; however, in the presence of the GHB precursor, BTD (2 mM), and the GHB isomer, BHB (2 mM), the uptake of GHB was not changed significantly (Fig. 6C). Two MCT inhibitors, phloretin and CHC, significantly decreased the uptake of GHB in BLM vesicles (Fig. 6C).

**Cellular Uptake of GHB in Rat MCT1 Gene-Transfected Cells**

**pH and Sodium Effects on the Uptake of GHB.** The uptake of GHB by rat MCT1 gene-transfected cells and control cells at different pH values was determined and compared with that at pH 7.5. For all the pH values examined in the study, the uptake in rat MCT1 gene-transfected cells was
significantly higher than the uptake in control cells (Fig. 7A). The uptakes of GHB in rat MCT1 transfected cells at pH 5.5, 6.0, 6.5, and 7.0 are all significantly higher than that at pH 7.5, whereas the uptake at pH 8.0 is lower than that at pH 7.5 (Fig. 7A). However, the uptakes of GHB in control cells at pH 6.0, 6.5, 7.0, and 8.0 are all similar to that at pH 7.5, with only the uptake of GHB at pH 5.5 being significantly greater than that at pH 7.5 (Fig. 7A). We examined the effect of sodium on the uptake of GHB in rat MCT1 transfected cells and control cells at pH 6.0 and pH 7.5. The uptake of GHB at both pH values in transfected and control cell lines was not significantly different in the presence and absence of sodium ions (Fig. 7B).

**Time Course and Concentration Dependence of the Uptake of GHB.** The uptake of 5.7 nM [3H]GHB by rat MCT1 gene-transfected cells and control cells was examined over time, up to 1 h. The uptake was linear up to 10 min, and 1 min was chosen for initial uptake rate determination (Fig. 7C) to minimize the possible metabolism of GHB in this cell line. The uptake of various concentrations of GHB from 0.1 to 30 mM, at an external buffer pH of 6.0, was determined. The uptake of GHB in rat MCT1 gene-transfected cells exhibited typical Michaelis-Menten type kinetics, and the uptake of GHB in control cells exhibited passive diffusion with no saturation observed at high concentrations (Fig. 7D). The uptake data obtained in rat MCT1 gene-transfected cells were fitted to eqs. 1 and 2. We also determined the net uptake values for GHB in rat MCT1 gene-transfected cells by subtracting the control cell values and fitted the net uptake data with eqs. 1 and 2. The total uptake values were best fitted with eq. 2, and the net uptake data were best fitted with eq. 1. The $K_m$ and $V_{max}$ values obtained from the two fitting were similar, suggesting the presence of only one transporter for GHB uptake by rat MCT1 gene-transfected cells. The kinetic parameters of GHB by rat MCT1 gene-transfected cells are $K_m$ of 4.6 ± 1.5 mM and $V_{max}$ of 81.2 ± 36.8 nmol/mg/min or 1350 ± 613 pmol/mg/s (Table 1).

**Effects of Inhibitors on GHB and D-Lactate Uptake.** The potential inhibitory effects of selected MCT inhibitors on GHB (5.7 nM) uptake were examined at pH 6.0, following incubation with inhibitors at various concentrations for 1 min. The uptake results were compared with the values in the absence of any inhibitor. TEA was used as a negative control, and 5 mM GHB was used as a positive control. The results demonstrated that GHB uptake was significantly inhibited by D-lactate (5 mM), α-CHC (2 mM), phloretin (0.1 mM), probenecid (0.1 mM), and salicylic acid (2 mM), but not TEA (2 mM) (Fig. 7E). Likewise, D-[3H]lactate (25 nM) uptake by cells was significantly inhibited by GHB (5 mM), α-CHC (2 mM), and phloretin (0.1 mM), but not by TEA (2 mM) (Fig. 7F). We examined the effect of the MCT inhibitors α-CHC and phloretin on GHB uptake by control cells but found no significant effect (data not shown).

**Discussion**

GHB is a four-carbon chain monocarboxylic acid with a $pK_a$ of 4.7. At physiological pH, GHB is almost completely ionized and not readily permeable across cell membranes. In this study, we demonstrated that the transport of GHB in rat kidney is carrier-mediated and inhibited by the MCT substrates l-lactate and pyruvate; this agrees with the findings...
in our previous in vivo study (Morris et al., 2005) where we demonstrated that the renal reabsorption of GHB was concentration-dependent, with renal clearance increasing as steady-state plasma concentrations increased. The renal clearance was significantly increased by concomitant infusion of MCT substrates L-lactate and pyruvate (Morris et al., 2005). Carrier-mediated transport of GHB has also been demonstrated in the intestine (Arena and Fung, 1980), in brain tissue (Benavides et al., 1982), and at the blood-brain barrier (Bhatacharya, 2004). There is limited information available regarding the transport mechanism of GHB in renal proximal tubules.

In this study, we demonstrated the pH-dependent transport of GHB and L-lactate in both BBM and BLM vesicles isolated from rat kidney cortex. These findings are consistent with the pH-dependent transport of L-lactate by members of the MCT family. Using BLM vesicles from medullary thick ascending limb, Eladari et al. (1999) demonstrated a pH-dependent transport of L-lactate but no pH-dependent transport of L-lactate in BBM vesicles. These differences may reflect differences in the membrane vesicle preparations or other experimental differences. The interesting finding of pH-dependent transport of L-lactate was confirmed by inhibitor studies, where a MCT inhibitor, CHC, could significantly decrease the L-lactate uptake. Studies with GHB uptake also revealed similar pH dependence in uptake into BBM vesicles. Uptake values were normalized by protein concentration. GHB was used at concentration of 40 μM. Concentrations of the inhibitors are the same as given in the legend for Fig. 2. Results presented as mean ± S.D., n = 6 to 12. *, p < 0.05, **, p < 0.01, one-way ANOVA followed by Dunnett’s test.

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BBM</th>
<th>BLM</th>
<th>MCT1 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>8.0 ± 1.8</td>
<td>10.5 ± 2.6</td>
<td>4.6 ± 1.5</td>
</tr>
<tr>
<td>$V_{max}$ (pmol/mg/sec)</td>
<td>838 ± 48</td>
<td>806 ± 253</td>
<td>1350 ± 613</td>
</tr>
</tbody>
</table>

Fig. 4. Uptake of GHB in BBM vesicles: effect of pH, concentration, and inhibition. A, effect of buffer pH on the uptake of GHB in BBM vesicles. B, concentration-dependent uptake of GHB in BBM vesicles at pH 5.5 and 7.5. C, concentration-dependent uptake of GHB in BBM vesicles in the presence and absence of sodium. Closed circles, uptake in the presence of sodium; triangles, uptake in the absence of sodium; open circles, sodium-dependent net uptake obtained by subtracting the value in the absence of sodium from that obtained in the presence of sodium. D, effects of inhibitors on the uptake of GHB in the presence of a pH gradient (inside pH of 7.5, outside pH of 5.5) in BBM vesicles. E, effects of inhibitors on the uptake of GHB in the presence of a sodium gradient in BBM vesicles.
Fig. 5. Time course of GHB uptake in BLM vesicles under various conditions. A, time course of sodium-dependent uptake of GHB. Closed circles, uptake in the presence of sodium. Open circles, uptake in the absence of sodium. B, time course of pH-dependent uptake of GHB. Open circles, uptake at pH 7.5; closed circles, uptake at pH 5.5. C, time course demonstrating the effect of preincubation with bicarbonate (100 mM) on the uptake of GHB. Open circles, uptake in the absence of bicarbonate; closed circles, uptake in the presence of bicarbonate. Uptake values were normalized by protein concentration. Results presented as mean ± S.D., n = 3 to 4.

Fig. 6. Uptake of GHB in BLM: effect of pH and inhibitors A, effect of buffer pH on the uptake of GHB in BLM vesicles. B, concentration-dependent uptake of GHB in BLM vesicles at pH 5.5 and 7.5. Closed circles, uptake at pH 5.5; triangles, uptake at pH 7.5; open circles, pH-dependent net uptake obtained by subtracting the value at pH 7.5 from the value at pH 5.5; solid lines, lines obtained by fitting the data to eqs. 1 to 3, using Winnonlin 2.1. C, effects of inhibitors on the uptake of GHB in BLM vesicles in the presence of a pH gradient. GHB was used at concentration of 40 μM. Concentrations of the inhibitors are the same as given in the legend for Fig. 2. Uptake values were normalized by protein concentration. Results presented as mean ± S.D., n = 3 to 4. *p < 0.05; **p < 0.01; one-way ANOVA followed by Dunnett’s test.
MCT2 shares 60% identity with MCT1 but exhibits much limited tissue distribution to liver, brain, and kidney. MCT2 transports pyruvate with a much higher affinity than other MCTs (Lin et al., 1998), and it also transports \( L \)-lactate with a higher affinity (Lin et al., 1998) than MCT1 (Lin et al., 1999). Using rat MCT1 transfected cells, we demonstrated that GHB is a substrate of MCT1 and is transported in a pH-dependent manner but not a sodium-dependent manner.

**Fig. 7.** Cellular uptake of GHB in MCT1 gene-transfected cells and control cells. A, pH-dependent uptake by rat MCT1 gene-transfected cells (black bars) and control cells (white bars). All the values are compared with those for pH 7.5 for each group. B, effect of sodium on the uptake of GHB by rat MCT1 gene-transfected cells (black bars) and control cells (white bars). Uptake at pH 7.5 by control cells was used as the control. C, time course of uptake of GHB at pH 6.0 by rat MCT1 gene-transfected cells and control cells (open circles). D, concentration-dependent uptake by rat MCT1 gene-transfected cells and control cells. Closed circles, uptake by rat MCT1 gene-transfected cells; triangles, uptake by control cells; open circles, net uptake obtained by subtracting the value obtained from control cells from the value obtained from rat MCT1 gene-transfected cells; solid lines, lines obtained by fitting the data to eqs. 1 to 3 using Winnonlin 2.1. E, effects of inhibitors on the uptake of GHB by rat MCT1 gene-transfected cells at pH 6.0. F, effects of inhibitors on the uptake of \( D \)-lactate by rat MCT1 gene-transfected cells at pH 6.0. Uptake values were normalized by protein concentration. Results presented as mean ± S.D., \( n = 3 \) to 4. *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \), one-way ANOVA followed by Dunnett’s test.
dependent manner. The similarity between the $K_{m}$ value obtained from BLM vesicles and that obtained from rat MCT1 transfected cells, and the similarity between the inhibitory effects on uptake by BLM vesicles and rat MCT1 transfected cells, suggest that rat MCT1 may represent an important transporter for GHB in BLM vesicles. We were only able to identify a single transport process using Eadie-Hofstee plot or Lineweaver-Burk plot analysis, although it is possible that rat MCT2 is also involved in the transport of GHB in BLM vesicles. The $K_{m}$ of GHB in BLM vesicles is close to that in BBM vesicles; whether this suggests that MCT1 is the sole transporter in two membranes is speculative. The $V_{max}$ values for pH-dependent transport were similar in both BBM and BLM, despite the much lower expression of MCT1 in BBM. This might suggest differences in activity due to differential posttranslational modifications in MCT1 or the contribution of other transporters in BBM, including other MCT members as well as sodium-dependent transporters. MCT4 and MCT6 are present in mammalian kidney; however, their distributions to the renal tubular cell membrane transport of monocarboxylates have not been characterized (Price et al., 1998).

In agreement with the literature, we found that the transport of l-lactate and GHB in BBM vesicles from rat kidney cortex was also sodium-dependent and that this sodium-dependent transport could be inhibited by acetocetate, pyruvate, and d-lactate. Jorgensen and Sheikh (1985) used BBM vesicles from rabbit kidney to demonstrate sodium-dependent common transport systems for ketone bodies including γ-hydroxybutyrate. The authors reported two sodium-driven electrogenic processes from whole cortex, pars convoluta, and pars recta, one of high affinity and the other of low affinity. Other reports in the literature have also confirmed the presence of a sodium-dependent transport process for l-lactate or pyruvate in mammalian kidney (Bara-Nieto et al., 1980; Nord et al., 1982; Mengual et al., 1989). Recently, the high-affinity sodium-dependent transport system for monocarboxylates has been identified as SLC5A8, a facilitated glucose transporter family member (Li et al., 2003; Miyauchi et al., 2004), and the low-affinity sodium-dependent transporter for monocarboxylates as SLC5A12 (Srivas et al., 2005). However, the transport of GHB by these sodium-dependent transporters has not been examined. The sodium-dependent uptake of GHB in BBM vesicles could also be significantly inhibited by pyruvate and d-lactate, which suggests that these transporters may also be involved in the reabsorption of GHB in the kidney at the BBM.

It has also been suggested that an anion exchanger is present in both luminal and basolateral renal membranes (Talor et al., 1987), and previous studies in red blood cells have identified an anion exchange transporter important in the transport of l-lactate (Deuticke et al., 1982). However, the current study demonstrated that preloading with bicarbonate had no effect on GHB uptake by BBM and BLM vesicles, and the inhibitor for the anion exchanger, DIDS, did not inhibit the GHB uptake by BBM and BLM vesicles under either a pH gradient or sodium gradient (data not shown). This suggests that anion exchange may not represent a significant mechanism for GHB transport in rat kidney.

This study provides a mechanistic explanation for our previous in vivo study where we found that l-lactate and pyruvate could increase the renal clearance of GHB by inhibiting its renal reabsorption. This inhibition resulted in an increase in the overall clearance of GHB in rats after the administration of high doses, such as would be obtained following overdoses of GHB. Inhibition of the renal reabsorption of GHB with MCT inhibitors or substrates represents a potential strategy for increasing the elimination of GHB following GHB intoxication. The present study provides the first report on the carrier-mediated transport of GHB in both BBM and BLM of rat kidney cortex. Under our experimental conditions and at physiological pH, the majority of GHB molecules will be ionized, and its renal reabsorption in BBM and BLM is mediated by specific transporters; in our studies, nonionic diffusion was minimal under all conditions (less than 15%; data not shown). In summary, we found that similar to the renal transport of l-lactate, GHB is transported at the BBM in a pH- and sodium-dependent manner and at the BLM by a pH-dependent process; rat MCT1 and MCT2 are present in the BLM and a lower amount of MCT1, but no MCT2 is present in the BBM; and GHB is a substrate for rat MCT1. These findings lend insight into the physiological processes underlying GHB reabsorption in the kidney and provide mechanistic strategies to increase the clearance of GHB following overdoses of this drug of abuse.

Acknowledgments

We thank Akri Tsuji and Ikumi Tamai for providing the MDA-MB231-MCT1 cells. We also thank Daniel Brazeau and Paul Sanders for assistance with the RT-PCR experiment.

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