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Research Paper

Monocarboxylate Transporter (MCT) Mediates the Transport of γ-Hydroxybutyrate in Human Kidney HK-2 cells

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Purpose. Previous studies in our laboratory have suggested that GHB may undergo renal reabsorption mediated by monocarboxylic acid transporters (MCT). The objectives of this study were to characterize the renal transport of GHB using HK-2 cells and the role of MCT in the renal transport of GHB.
Materials and Methods. Western blot was used to detect the protein expression of MCT1, 2, and 4. Cellular uptake and directional flux studies were conducted to investigate the transport of GHB and L-lactate. RNA interference assay was used to investigate the involvement of MCT isoforms in the transport of GHB.
Results. MCT1, 2 and 4 were present in HK-2 cells. The cellular uptake of L-lactate and GHB exhibited pH- and concentration-dependence (L-lactate: K_m of 6.5 ± 1.1 mM and V_{max} of 340 ± 60 nmol $mg^{-1}min^{-1}$; GHB: K_m of 2.07 ± 0.79 mM, V_{max} of 27.6 ± 9.3 nmol $mg^{-1}min^{-1}$, and a diffusional clearance of 0.54 ± 0.15 μl $mg^{-1}min^{-1}$), but not sodium-dependence. α-Cyano-4-hydroxycinnamate (CHC) competitively inhibited the uptake of GHB and L-lactate with inhibition constants (K_i) of 0.28 ± 0.1 mM, and 0.19 ± 0.03 mM, respectively. Using small-interference RNA (siRNA) for MCT1, the protein expression of MCT1 and the uptake of L-lactate and GHB were significantly decreased. The siRNA treatment of MCT2 in HK-2 cells inhibited the uptake of GHB by 17%, and the siRNA treatment of MCT4 demonstrated no inhibition of GHB uptake. GHB exhibited a directional flux across HK-2 monolayer from apical to basal chambers in the presence of a pH gradient of pH 6.0 to pH 7.4.
Conclusion. These data suggest that MCT1 represents an important transporter for GHB transport in renal tubule cells, responsible for the reabsorption of GHB in the kidney.

KEY WORDS: MCT; monocarboxylate transporters; renal transport; transporters; γ-hydroxybutyrate.

INTRODUCTION

As a naturally occurring short-chain fatty acid, γ-hydroxybutyric acid (GHB) is present in mammalian brain (1,2), as well as liver and kidney, where it is formed from γ-aminobutyric acid (3). The therapeutic uses of GHB include the treatment of the sleep disorder narcolepsy (4), and the treatment of alcohol dependence in Europe (5). GHB has also been used by body builders as a popular steroid alternative due to its growth hormone-releasing effects (6), and by drug-abusers as a recreational drug at nightclubs and rave parties for its euphoric effects (7). GHB has also been used as a means of drug-facilitated sexual assaults (date rape) due to its hypnotic/amnesic effects. The adverse effects associated with GHB are dose-dependent, including nausea, vomiting, dizziness, bradycardia, hypotension, coma, seizure and even death (8). There are no specific treatments for GHB overdoses; treatment generally consists of supportive care.

The pharmacokinetics of GHB has been reported to be non-linear in rats (9,10) and humans (11–14), with decreasing total clearance with increasing dose. The mechanisms underlying the non-linear pharmacokinetics of GHB include capacity-limited metabolism (9–12,15) and absorption of GHB (16). Recently, we found that the renal clearance of GHB also contributed to the non-linear pharmacokinetics of GHB (17). We hypothesize that the non-linear renal clearance of GHB may be due to its carrier-mediated renal reabsorption by monocarboxylic acid transporters (MCT), since the MCT substrates, L-lactate and pyruvate, could inhibit the active reabsorption and increase the renal clearance of GHB (17).

Among the 14 members of the MCT family, MCT 1–4 have been demonstrated to be proton-coupled MCTs (18,19), with similar, but not identical, substrates, including endogenous compounds, such as lactate, pyruvate, butyrate and ketone bodies (19–22) and exogenous compounds, such as

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ABBREVIATIONS: CHC, α-cyano-4-hydroxycinnamate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; GHB, γ-hydroxybutyrate; MCT, monocarboxylate transporter; PCMB, p-chloro-mercuribenzoic acid; TEA, tetraethylammonium chloride; SiRNA, small interference RNA.

foscarnet, nateglinide, simvastatin acid and lovastatin acid (23–26). MCT1, the first member identified, has been the most extensively characterized. MCT1 transports monocarboxylates in a pH-dependent manner with a moderate K_m for D-lactate in mM range, and a lower K_m for its L-isomer. The tissue distribution of MCT1 is ubiquitous, and includes, intestine, colon, muscle, heart, brain, kidney and red blood cells (18,19).

HK-2 cell is a human proximal tubular epithelial cell line that was immortalized with human papilloma virus E6/E7 genes (27). This cell line retains the functional characteristics of proximal tubular epithelium of human kidney (28). HK-2 cells can form a monolayer under culture conditions and polarize into apical and basal membranes (29). The cells retain functional transport activities of glucose transporters (27); other transporters have also been identified in this cell line, including P-glycoprotein (30–32) and MCTs (33). HK-2 cells have been employed to study active vectorial transport (34) and secretion of protein products (29).

The overall goal of this study is to characterize the renal transport mechanisms of GHB in order to identify strategies to increase its renal clearance following overdoses of the drug. The objectives of this study were to investigate the (1) transport driving forces, (2) transport kinetics and directional flux, (3) effect of various transport inhibitors, and (4) the role of MCT1, MCT2 and MCT4 of GHB in human kidney HK-2 cells.

MATERIALS AND METHODS

Materials

L-lactate, D-Lactate, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), α-cyano-4-hydroxycinnamate (CHC), p-chloro-mercuribenzoic acid (pCMB), salicylate butyrate, phloretin, rifampicin, probenecid, tetraethylamonium chloride (TEA) were purchased from Sigma (St Louis, MO). [2, 3-^{3}H]-γ-Hydroxybutyric acid (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), and L-[^{14}C (U)]-lactate (56 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Biodegradable counting scintillate was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Human Kidney-2 (HK-2) cells were purchased from the American Type Culture Collection (Manassas, VA).

Cell Culture

HK-2 cells were maintained at 37°C in 5% CO_2/95% air environment, as previously described (29). Cells were cultured in Dulbecco's Modified Eagle Medium/F12 (D-MEM/F12, Invitrogen Corp. Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units penicillin and 100 μg/ml streptomycin. Cells reached confluence after 5–7 days in culture. They were harvested for studies between passage numbers 13 and 30. Cells were subcultured at a ratio of 1:4 using 0.05% trypsin and 0.53 mM EDTA. Cells were seeded in 35 mm (diameter) plastic culture dishes two days before uptake studies. For the directional flux study, HK-2 cells were maintained on Transwell® polycarbonate permeable supports (Corning Inc. Acton, MA) as previous described (29), with fresh medium changed daily for 7–10 days.

Western Blot Analysis

The expression of gamma-glutamyl transpeptidase (GGT), Na^+/K^+-ATPase, MCT1, MCT2 and MCT4 in HK-2 cells was examined by Western blot. HK-2 cells were harvested and lysed with lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X100, 10 mM Tris-Cl pH 7.4, 5 mM DTT, 100 μM phenylmethanesulfonyl fluoride (PMSF) in isopropanol, 5 mM ε-aminocaproic acid) on ice for 30 min and then centrifuged at 16,000 g for 18 min at 4°C. The supernatants were collected and separated on 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS and then transferred to nitrocellulose membrane. Membranes were blocked by 5% (w/v) milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.05% (v/v) Tween 20 at 20°C for 1 h. The membranes were incubated with primary antibodies for MCT1 (1 μg/ml), MCT2 (1 μg/ml) and MCT4 (1 μg/ml) (US Biological, Swampscott, MA) overnight at 4°C. The membranes were then incubated with specific horseradish peroxidase-conjugated secondary antibodies (Chemicon, Temecula, CA) for 1 h at room temperature. Immunoblots were developed with the enhanced chemiluminescence system (ECL) (Amersham International, UK).

Uptake Studies

Growth medium was removed from the cell monolayers and cells were washed three times with uptake buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.4). One milliliter of buffer containing 0.05 μCi of [^{14}C]-L-lactate or 0.5 μCi [^{3}H]-GHB was added to the dishes. For time-dependent studies, cells were incubated at room temperature for 0.5, 1, 5, 10, 15, 30, 60 and 120 min. From these studies a time-point of 10 min was chosen to represent the linear uptake of GHB and 1 min to represent the linear uptake of L-lactate. For the pH-dependent study, the cells were incubated with uptake buffer with different pH values (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.4), while for the sodium-dependent study, the cells were incubated with sodium buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.4) or sodium free buffer (137 mM N-methyl-D-glucamine, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.4). Inhibitors used in cellular uptake studies included salicylate, CHC, pCMB, DIDS, probenecid, rifampicin, TEA, phloretin, L-lactate, D-lactate, and butyrate. The uptake was stopped by aspirating off the buffer, and the cells were washed immediately three times with ice-cold stop buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.4). The cells were solubilized by 1 ml lysis buffer containing 0.3 N NaOH and 1% SDS, and the cell lysates were collected from the dishes after 1 h. The radioactivity was determined by mixing 3 ml scintillation liquid with 200 μl of cell lysate and counted with a liquid scintillation counter (1,900 CA, Tri-carb liquid scintillation analyzer, Packard Instrument Co. Downers Grove, IL). Protein concentrations were determined by the Bicinchoninic Acid protein assay kit (BCA, Pierce Chemicals, Rockford, IL) with bovine serum albumin as standard. The

results were normalized for the protein content of the cells in each dish and accumulation was expressed as pmol or nmol mg protein^{-1}min^{-1}.

Flux of GHB Across HK-2 Monolayers

The transport experiments using HK-2 cell monolayers were conducted as previously described (33,35). The metabolism of L-lactate in HK-2 cells is appreciable if cells are incubated for periods of time longer than 1 min, but the metabolism of GHB in HK-2 cells is minimal at incubation times up to 30 min (data not shown). Therefore GHB, but not L-lactate, was used to investigate the transcellular flux. Briefly, the cells were seeded in six-well plates with polycarbonate transwell inserts (Corning Inc., Corning, NY). After 7–10 days culture, the cells were rinsed and incubated with uptake buffer for 10 min. The trans-epithelial-resistance (TEER) was measured and those wells with TEER values over 600 Ω were used in the studies. Transport buffer (similar to previous uptake buffer but with 5 mM D-glucose) containing 5.7 nM ^{3}H-GHB was loaded in the apical (1.5 ml) or basal (2.6 ml) chamber. The transport studies were conducted at different pH values for apical or basal chambers at room temperature, with or without the MCT inhibitor CHC. The pH combinations used in this experiment are listed in Table II. Samples of 100 µl were removed from the receiving chamber at 15, 30, and 60 min and replaced with the same volume of fresh transport buffer. The apparent permeability of ^{3}H-mannitol was also measured to ensure the integrity of cell monolayers. Among all the conditions tested, mannitol showed no apparent directional transport either from apical-to-basal (AP-BL) or basal-to-apical (BL-AP). The calculation of apparent permeability was carried out as previously described (35), and given in the following equation. $P_{app}=\frac{\Delta Q}{\Delta t}\times\frac{1}{A\times C_0}$ where $\Delta Q/\Delta t$ is the rate of appearance of radiolabelled substrates in the receiving chamber; C_0 is the initial concentration of the radiolabelled compound in the donor chamber; and A is the cell monolayer surface area (4.71 cm^2).

RNA Interference

Two siRNA constructs targeting MCT1 (exon5 and exon3, respectively), two siRNA constructs targeting MCT2 (exon4 and exon5, respectively), and two siRNA constructs targeting MCT4 (exon5), were designed using the manufacturer supplied tools and purchased from Ambion Inc. (Houston, TX). The sequences of siRNA for MCT1 were: (sense/antisense, 5′—3′) no. 1 GCAGUAUCCUGGU GAAUAAtt, UUAUUCACCAGGAUACUGCtg and no. 2 CGAAUAA AGAUAGGAUUGGtt, CCAAUCCUAU CUUUAUUCGtt. The sequences of siRNA for MCT2 were: no. 3 CCCUU GAGCAAAUCUAAACtt, GUUUA GAUUUGCUCAAGGtt. The sequences of siRNA for MCT4 were: no. 4 CGUCUA CAUGUACGUGUUCtt, GAACACGUACAUGUAGACGtg. The non-specific scrambled control RNA was also purchased from Ambion Inc. Cells were grown in six-well plates and were at 20–40% confluence one day before transfection. Cells were transfected with siRNA at a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen Corp. Carlsbad, CA), according to the manufacturer's instructions. The cells were then incubated at 37°C in 5% CO_2 for 48 h before the Western blot analysis and uptake experiments.

Data Analysis

The data are presented as mean ± SD. 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. The differences with a p value of 0.05 or less were considered as statistically significant. Data analysis was performed using GraphPad Prism (GraphPad Inc., San Diego CA). The transport kinetic parameters: Michaelis–Menten constant K_m and maximum uptake rate V_{max} were determined by fitting the data using weighted non-linear regression analysis (Winnolin 2.1 Pharsight Corp. Cary NC) and the following equations:

$$v=\frac{V_{max}*C}{K_m+C} \tag{1}$$

$$v=\frac{V_{max}*C}{K_m+C}+P*C \tag{2}$$

$$v=\frac{V_{max,1}*C}{K_{m,}1+C}+\frac{V_{max,2}*C}{K_{m,}2+C} \tag{3}$$

$$v_i=\frac{V_{max}*C_0}{K_m*(1+C_i/K_i)+C_0} \tag{4}$$

$$v_i=\frac{V_{max}*C_0}{K_m*(1+C_i/K_i)+C_0}+P*C_0 \tag{5}$$

Here v is the uptake rate of GHB or L-lactate and C is the concentration of GHB or L-lactate. P is the non-saturable uptake clearance. The goodness of fit was determined by the sum of the squared derivatives, the residual plot and the Akaike Information Criterion (AIC). The equation that provided the smallest CV% and AIC for the data was used to determine the K_m and V_{max} parameters for the uptake data.

RESULTS

Western Blot Results

We previously demonstrated that the mRNA for MCT1, MCT2, MCT3 and MCT4 was present in HK-2 cells, with MCT1 and MCT2 localized on the basolateral membrane (33). In this investigation, we confirmed that the protein of MCT1, MCT2 and MCT4 was present in HK-2 cells (Fig. 1). The staining of MCT4 protein was faint compared with MCT1 and MCT2.

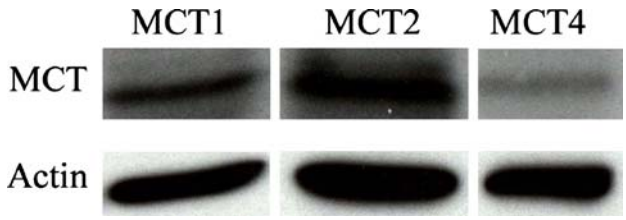


Fig. 1. Expression of MCT isoforms in HK-2 cells. *Upper lanes* are the MCT isoform protein, MCT1 (~42 KD), MCT2 (~37 KD) and MCT4 (~50 KD); *lower lanes* are β-actin, used as the loading control.

Uptake of L-lactate in HK-2 Cells

Na^+ and pH effect. Uptake of L-lactate by HK-2 cells was a pH-dependent process, and the uptake rate was significantly increased with decreasing buffer pH (Fig. 2a). To examine the Na^+ effect on L-lactate uptake, transport was characterized in the absence or presence of a sodium-containing buffer at pH 6.0 and pH 7.5. At pH 6.0, the uptake of L-lactate was not affected by the presence or absence of Na^+, while at pH 7.5, a significant reduction was observed in the absence of sodium (Fig. 2a). However, the uptake of L-lactate can be inhibited to the same level when using the classical MCT inhibitor CHC at the two different pH values (Fig. 2a).

Kinetics of L-lactate uptake and the effects of inhibitors on L-lactate uptake. The uptake of L-lactate by HK-2 cells was linear up to 10 min (Fig. 2b), and a 1 min uptake time was chosen for the concentration dependent study. Concentration-dependence of L-lactate uptake was demonstrated in this study, and the uptake was best fitted by Eq. 1 which suggested a single transporter mediated transport process with a K_m of 6.5 ± 1.1 mM, and V_{max} of 340 ± 60 nmol·mg^{-1}min^{-1} (Table I and Fig. 2c). An Eadie–Hofstee plot of the concentration-dependent data was linear, which suggested that a one-transporter model was suitable (data not shown). The inhibition of L-lactate uptake by CHC was competitive in nature, and a CHC concentration-dependent inhibition of L-lactate uptake was demonstrated (Fig. 2e). Based on the model fitting criteria AIC and CV%, this inhibition profile was best fitted with Eq. 4 with a K_i of 0.19 mM (Fig. 2e). GHB and the MCT inhibitors CHC (2 mM), phloretin (0.5 mM) and pCMB (0.5 mM) could significantly inhibit the uptake of L-lactate (0.45 μm) (Fig. 2d).

Uptake of GHB by HK-2 Cells

Na^+ and pH effect. Uptake of GHB by HK-2 cells was a pH-dependent process, and the uptake rate was significantly increased with decreasing buffer pH, with the highest uptake value observed at pH 5.5 (Fig. 3a). To examine the Na^+ effect on GHB uptake, transport was characterized in the absence or presence of a sodium-containing buffer at pH 6.0 and pH 7.5. At pH 6.0, the uptake of GHB was not affected by the presence or absence of Na^+, while at pH 7.5, a significant reduction was observed in the absence of sodium (Fig. 3b). However, the uptake of GHB could be inhibited to the same level when using the classical MCT inhibitor CHC at the two different pH values (Fig. 3b).

Kinetics of GHB uptake and the effects of inhibitors on GHB uptake. The time course of the uptake of GHB by HK-2 cells demonstrated an overshoot phenomenon, which suggested a transporter-mediated uptake process (Fig. 3c). Concentration-dependence of GHB uptake was demonstrated in this study, and the uptake rate was best fitted by Eq. 2 which suggested a single transporter-mediated transport process with a K_m of 2.07 ± 0.79 mM, V_{max} of 27.6 ± 9.3 nmol·mg^{-1}min^{-1}, and diffusional clearance of 0.54 ± 0.15 μl·mg^{-1}min^{-1}, respectively, (Table I and Fig. 3d). An Eadie–Hofstee plot of the concentration-dependent data also gave a biphasic curve (data not shown). The inhibition of GHB uptake by CHC was competitive in nature (Fig. 4b), and a CHC concentration-dependent inhibition on GHB uptake was demonstrated (Fig. 4c). Based on the model fitting criteria AIC and CV%, this inhibition profile was best fitted with Eq. 5 with a K_i value of 0.28 ± 0.10 mM, which also suggested a single transporter-mediated process. The uptake of GHB (0.1 mM) was significantly inhibited by MCT1 substrates butyrate (2 mM), D-lactate (2 mM), and L-lactate (2 mM). MCT inhibitors, phloretin (0.5 mM), pCMB (0.5 mM), and CHC (2 mM) significantly inhibited the uptake of GHB to about 45% or more of the control. The OAT inhibitors, probenecid (0.5 mM) and salicylate (2 mM), and the anion exchanger inhibitor DIDS (2 mM but not 0.1 mM) also inhibited the uptake of GHB by HK-2 cells; however, the OATP inhibitor rifampicin (0.5 mM) and the OCT inhibitor TEA (5 mM) had no effect on the uptake of GHB (Fig. 4a).

Effects of siRNA Treatments on Protein Expression and Activity

Transfection of HK-2 cells with the two siRNA for MCT1 significantly decreased the protein expression of MCT1 as examined by Western blot analysis (Fig. 5a), with no. 1 siRNA treatment completely inhibiting MCT1 protein expression. Using ^{14}C-L-lactate as substrate, the uptake was significantly suppressed in the siRNA treatment group, with no. 1 siRNA being more potent. Using ^{3}H-GHB as the substrate, the uptake of GHB by HK-2 cells treated with siRNA for MCT1 was inhibited up to 70% of that by negative control cells treated with scrambled RNA. Transfection of HK-2 cells with siRNA for MCT2 could totally inhibit the protein expression of MCT2 (Fig. 6a). Transfection of HK-2 cells with siRNA for MCT4 could inhibit the protein expression significantly compared to that of the negative control, which was treated with a scramble siRNA (Fig. 6b). Uptake of L-lactate by HK-2 cells was significantly inhibited by the treatment of siRNA for MCT2, which is about 85% of that of the negative control (Fig. 6c). Uptake of L-lactate by HK-2 cells treated with the siRNA for MCT4 was also significantly lower than that of the negative control (Fig. 6d). Transfection of the siRNA for MCT2 only inhibited the uptake of GHB by 17%, while the transfection of the siRNA for MCT4 did not inhibit the uptake of GHB at all (Fig. 6e). The combination of siRNA for MCT1 and siRNA for MCT2, and the combination of siRNA for MCT1 and siRNA for MCT4, could also significantly decrease the uptake of GHB; however, the two combinations did not further decrease the uptake of GHB compared with the treatment of siRNA for MCT1 alone (Fig. 6f).

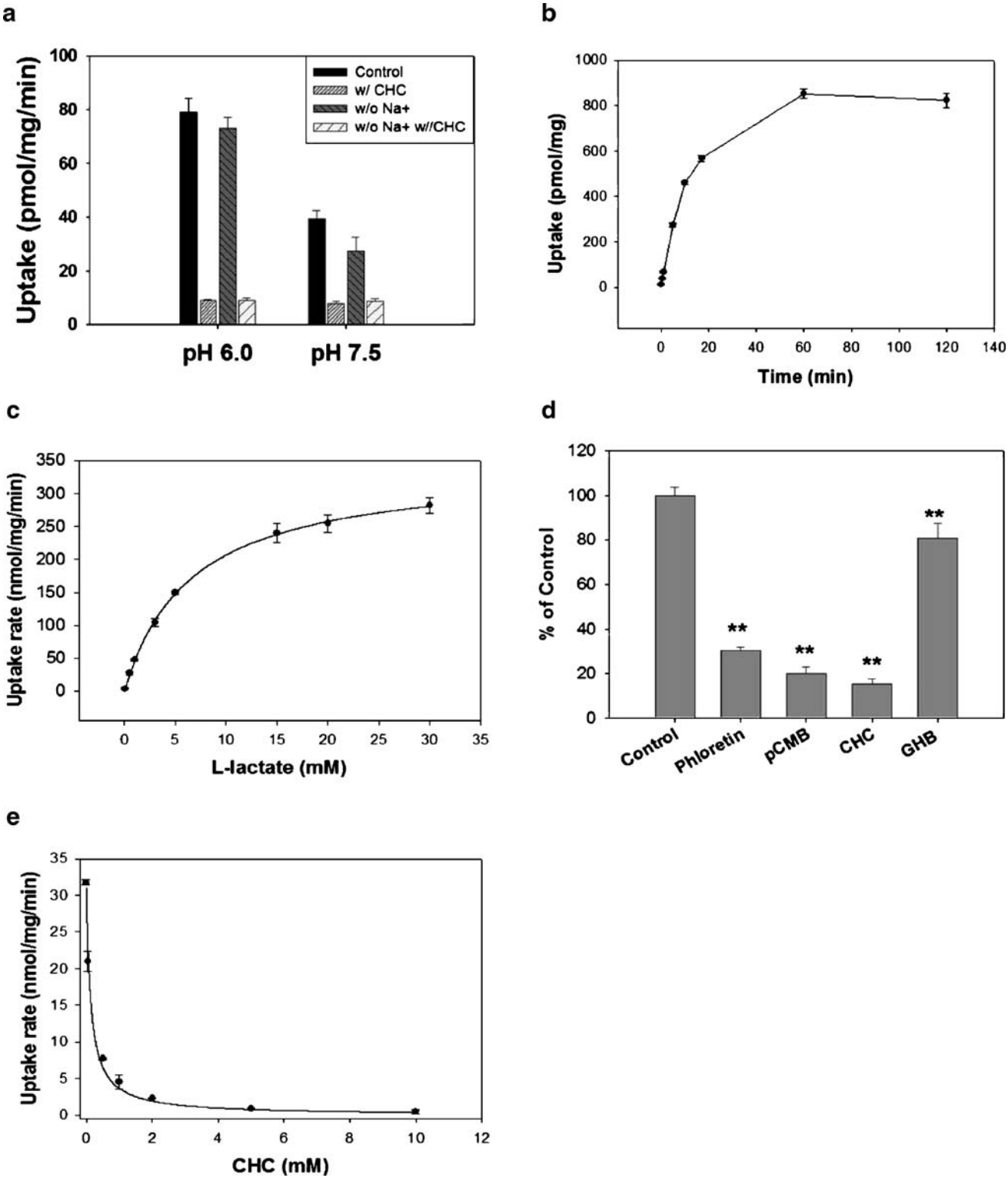


Fig. 2. Uptake of L-lactate in HK-2 cells: driving forces, concentration-dependence, effects of inhibitors, and concentration-dependent inhibition of CHC. (**a**) pH and sodium effect on the uptake of L-lactate; (**b**) time course of L-lactate uptake at pH 6.0; (**c**) concentration-dependent uptake at pH 6.0 with the *lines* representing the fitted results; (**d**) effects of CHC (2 mM), pCMB (0.5 mM), phloretin (0.5 mM), and GHB (2 mM) on the uptake of L-lactate (0.45 μM); (**e**) concentration-dependent inhibition of GHB uptake by CHC. Data are presented as mean ± SD, $n = 9–12$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table I. Kinetic Parameters for the Uptake of L-lactate and GHB in HK-2 Cells

Parameters	L-lactate	GHB
K_m (mM)	6.5 ± 1.1	2.07 ± 0.79
P (μl $mg^{-1}min^{-1}$)		0.54 ± 0.15
V_{max} (nmol $mg^{-1}min^{-1}$)	340 ± 60	27.6 ± 9.3
K_i (mM)	0.19 ± 0.03	0.28 ± 0.10

The K_i values represent the inhibition constant of CHC for the uptake of GHB or L-lactate, and were determined by non-linear regression analysis. All the values are presented as mean ± SD, $n = 3$–4.

Directional Flux of GHB Across HK-2 Monolayers

The basal membrane localization of MCT1 in HK-2 cells has been demonstrated previously (33). In this study, the function of MCT was also confirmed using L-lactate as a substrate. However, the extensive metabolism of L-lactate in cells prohibits its use as a candidate for transmembrane transport experiments, since these studies are performed over longer periods of time than the uptake studies. The transmembrane flux of GHB when pH 7.5 buffer was added to both chambers demonstrated little polarized transport, with a permeability ratio of BL-AP to AP-BL of 1.45 (Table II, Fig. 7). However, the transmembrane flux of GHB when pH 6.0 buffer was added to the apical chamber and pH 7.5 buffer was added to the basal chamber demonstrated a polarized transport, with a greater transport rate in the AP-BL direction than in the BL-AP direction (Table II, Fig. 7). This pH gradient from apical to basal chambers represents the physiological pH conditions that may exist in kidney tubules. If we reverse the pH gradient, with an apical buffer pH of 7.5 and a basal chamber buffer pH of 6.0, the directional flux was reversed, with a greater transport rate in the BL-AP direction compared with the AP-BL direction (Table II). When the buffer pH of both chambers was adjusted to pH 6.0, the directional flux was also reversed, with a greater transport rate in the BL-AP direction than in the AP-BL direction (Table II). This reversed directional flux could be eliminated by the MCT inhibitor, CHC (Table II).

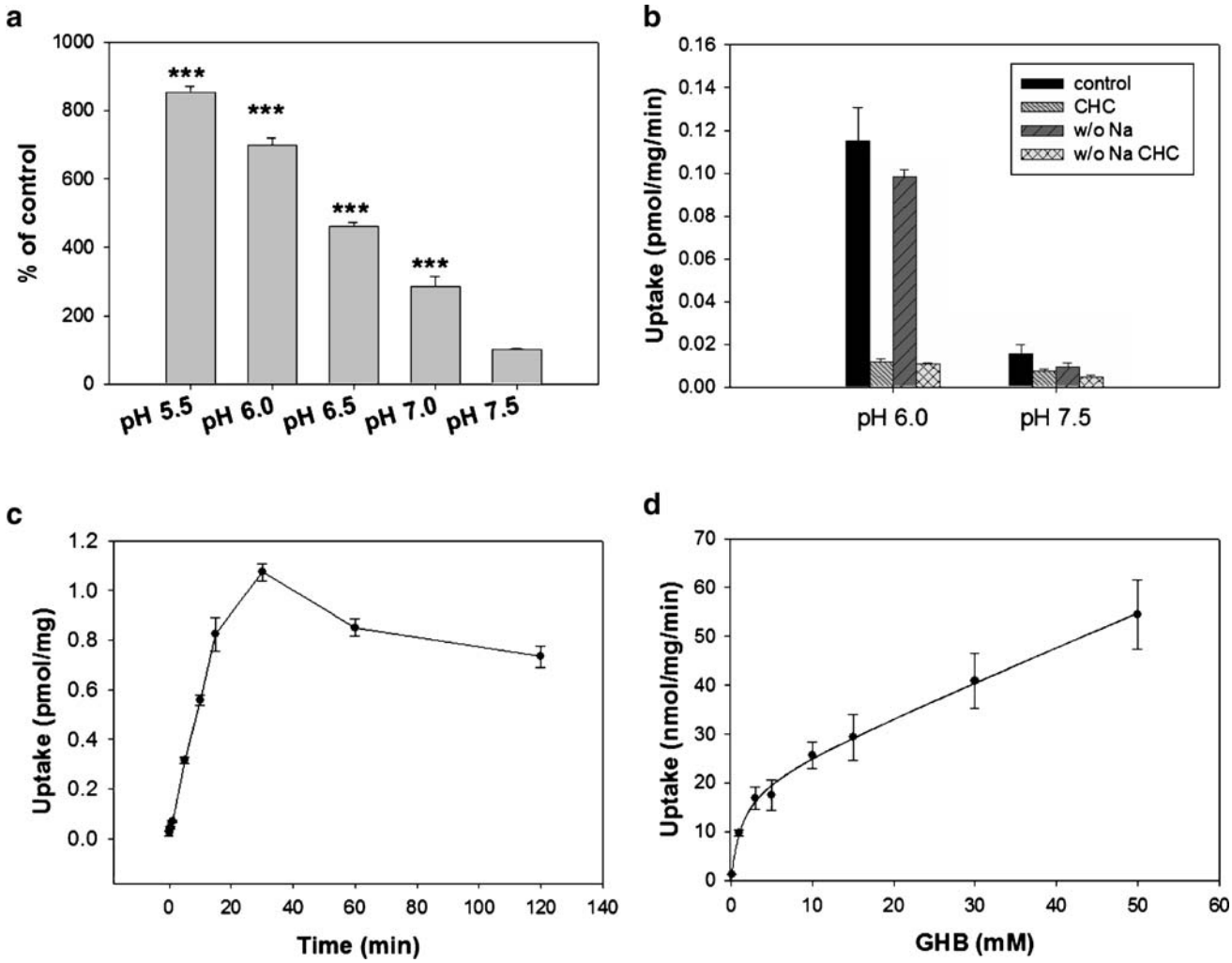
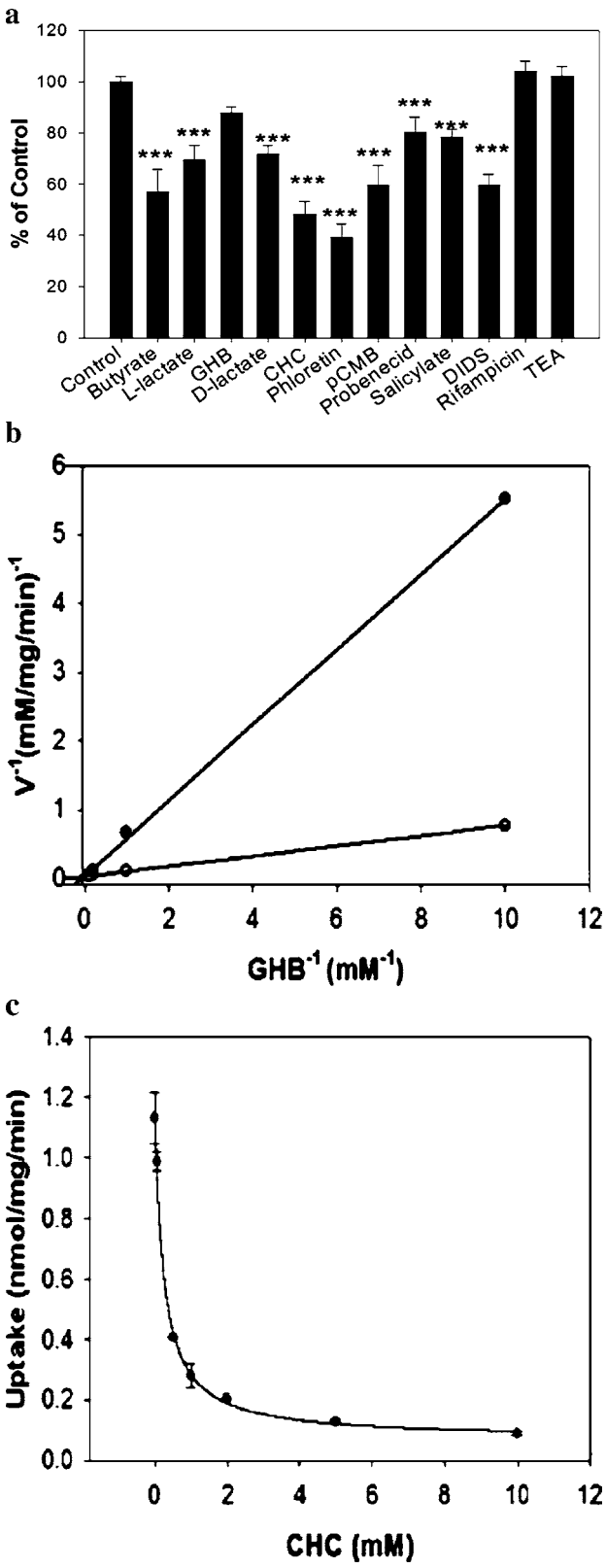


Fig. 3. Uptake of GHB in HK-2 cells: driving forces and concentration-dependence. (**a**) pH-dependent uptake of GHB, all the uptake values were compared to that at pH 7.5; (**b**) effect of sodium ions on the uptake of GHB at pH 6.0 and pH 7.5; no significant difference detected between the uptake of GHB in the absence or presence of sodium at pH 6.0; (**c**) time-course of GHB uptake at pH 6.0; (**d**) concentration-dependent uptake of GHB determined at pH 6.0. Data are presented as mean ± SD, $n = 6$–12.



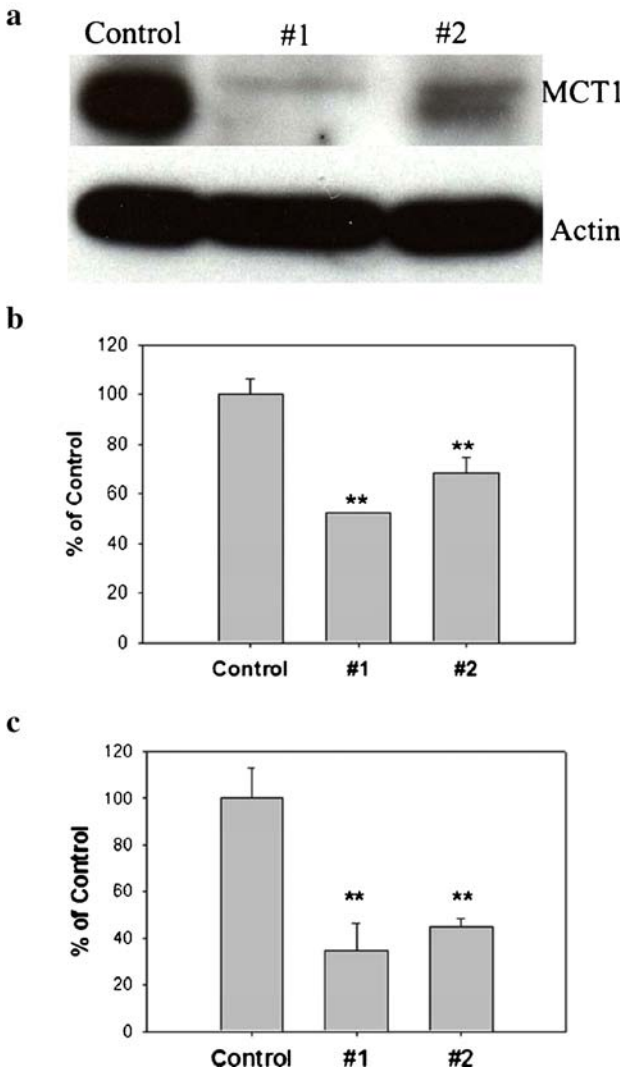


Fig. 5. Effects of siRNA for MCT1 in HK-2 cells. (**a**) MCT1 protein expression after treatment of HK-2 cells with two siRNA for MCT1, with β-actin used as loading control; (**b**) Uptake of GHB at pH 6.0 in siRNA-treated HK-2 cells; (**c**) Uptake of L-lactate at pH 6.0 in siRNA-treated HK-2 cells. No. 1 and no. 2 were two siRNA used in the study, as described in the Methods section. Data are presented as mean ± SD, $n = 6$–9. $^{**}P < 0.01$.

◀**Fig. 4.** Uptake of GHB in HK-2 cells: effects of inhibitors and the inhibition mechanism of CHC. (**a**) effects of CHC (2 mM), L-lactate (2 mM), D-lactate (2 mM), butyrate (2 mM), salicylate (2 mM), TEA (2 mM), DIDS (2 mM), phoretin (0.5 mM), pCMB (0.5 mM), rifampicin (0.5 mM) and probenecid (0.5 mM) on the uptake of GHB (0.1 mM) in HK-2 cells; (**b**) Lineweaver–Burk plot of uptake of GHB in the presence or absence of 2 mM CHC; *open circles* represent uptake in presence of CHC, and *closed circles* represent uptake in absence of CHC; the *lines* represent the fitted data; (**c**) concentration-dependent inhibition of GHB (0.1 mM) uptake by CHC in HK-2 cells at pH 6.0; the *lines* represent the fitted data. Data presented as mean ± SD, $n = 9$–12. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

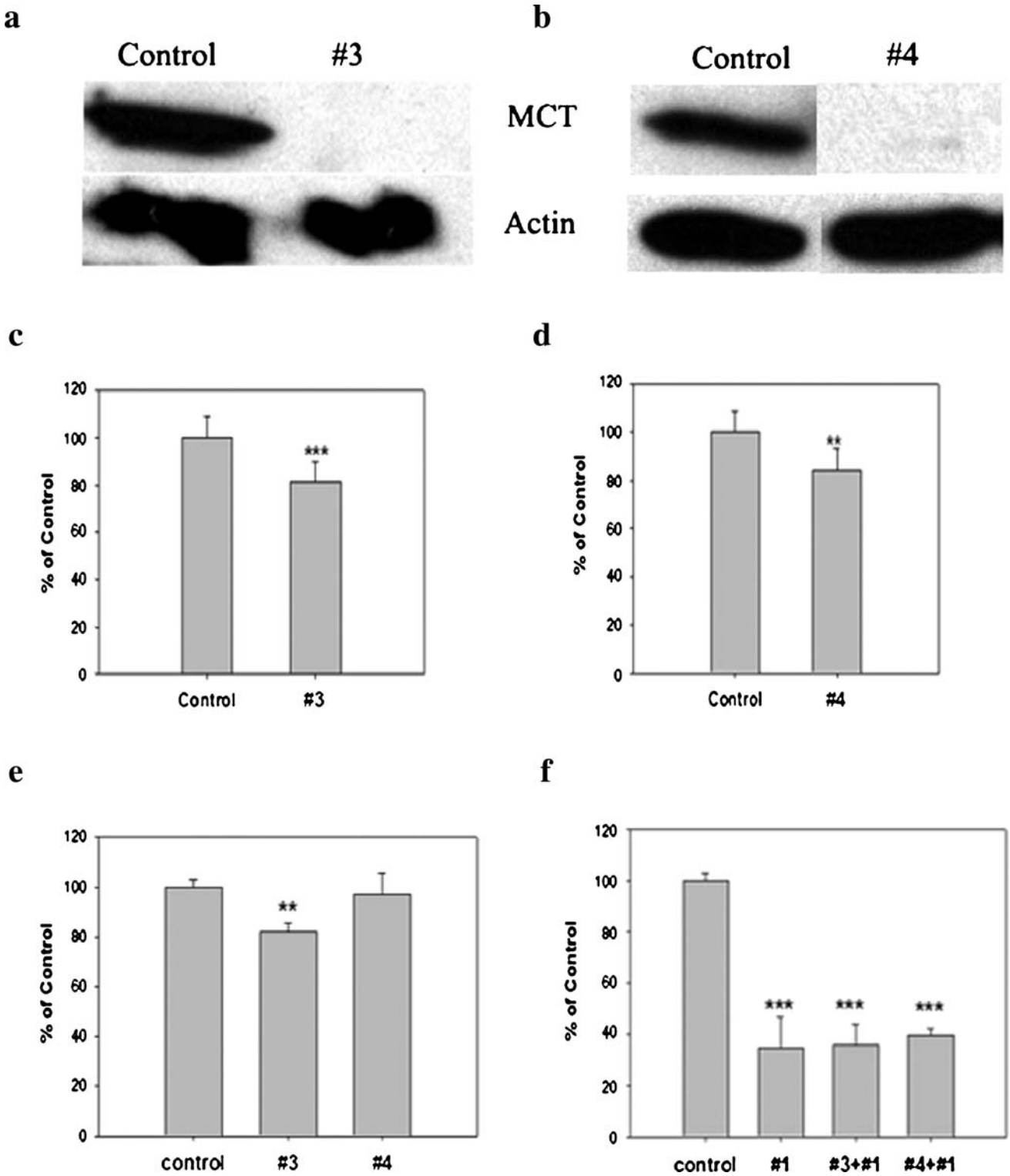


Fig. 6. Effects of siRNA for MCT2 and MCT4 in HK-2 cells. (**a**) MCT2 protein expression after treatment of HK-2 cells with two siRNAs for MCT1, with β-actin used as loading control; (**b**) MCT4 protein expression after treatment of HK-2 cells with two siRNAs for MCT1, with β-actin used as loading control; (**c**) Uptake of L-lactate in MCT2 siRNA-treated HK-2 cells; (**d**) Uptake of L-lactate in MCT4 siRNA-treated HK-2 cells; (**e**) Uptake of GHB in MCT2 siRNA-treated (no. 3) or MCT4 siRNA-treated (no. 4) HK-2 cells; (**f**) Uptake of GHB in HK-2 cells treated with siRNA for both MCT1 and MCT2, or for both MCT1 and MCT4. No. 1, no. 3 and no. 4 refer to the siRNA used in the study, with no. 1 representing siRNA for MCT1, no. 3 for MCT2 and no. 4 for MCT4. All the uptake studies were conducted at pH 6.0, room temperature. Data are presented as mean ± SD, $n = 6–9$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table II. Permeability Ratios for GHB Flux Across HK-2 Monolayers

	AP = 6.0 BL = 6.0	AP = 6.0 BL = 7.5	AP = 7.5 BL = 7.5	AP = 7.5 BL = 6.0	AP = 6.0 w/CHC BL = 6.0 w/CHC
$P_{app,\ AB}$ ($\times 10^5$ cm• s^{-1})	0.45 ± 0.14	1.63 ± 0.50	1.11 ± 0.37	0.44 ± 0.16	0.80 ± 0.23
$P_{app,\ BA}$ ($\times 10^5$ cm• s^{-1})	1.24 ± 0.21	0.95 ± 0.24	1.57 ± 0.35	1.32 ± 0.23	0.94 ± 0.10
Ratio	2.92 ± 1.90*	0.63 ± 0.23**	1.45 ± 0.15	3.19 ± 0.74**	1.22 ± 0.27

AP represents the buffer pH of the apical chamber of the transwell insert; BL represents the buffer pH of the basal chamber of the transwell insert. All data are presented as mean ± SD, $n = 3$–4.
*$P < 0.05$, **$P < 0.01$, by t-test. All the ratios were compared to group with both apical and basal buffer pH of 7.5. $P_{app,\ AB}$, apparent apical-to-basal permeability; $P_{app,BA}$, apparent basal-to-apical permeability; Ratio is calculated as $P_{app,BA}/P_{app,AB}$.

The extent of paracellular transport was determined using ^{3}H-mannitol. The permeability ratio of ^{3}H-mannitol at pH 6.0 (1.10) was similar to that at pH 7.5 (0.90) and no polarized transport was observed. It should be noted that the permeabilities of ^{3}H-mannitol and GHB were in the range of 10^{-6} cm/s, which suggested that paracellular pathway may represent an important transport route of GHB in HK-2 cell monolayers. Indeed, this permeability value of ^{3}H-mannitol is much higher than that obtained in Caco-2 cells or MDCK cells.

When pH 6.0 buffers were added to both chambers, the cellular accumulation of GHB after dosing from the basal chamber was significantly higher than that after dosing from apical chamber (Fig. 8), consistent with the localization of MCT1 on the basal membrane. The cellular accumulation of GHB at pH 6.0 was also significantly higher than that at pH 7.5 or that in the presence of CHC (data not shown) at the basal membrane.

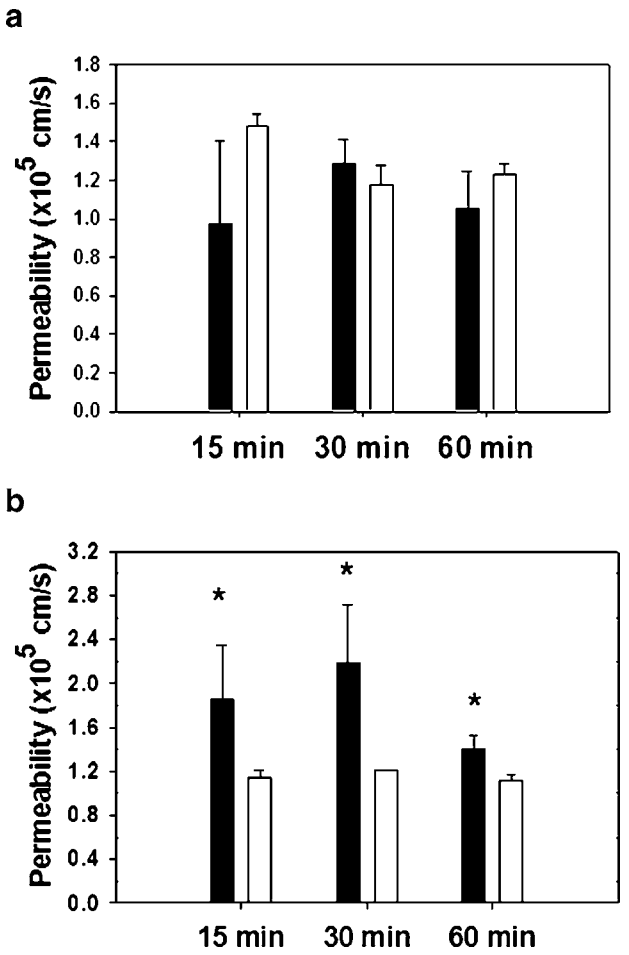


Fig. 7. Directional flux of GHB across HK-2 cell monolayers. (**a**) transport study conducted with pH 7.5 buffer for both apical and basal chambers; (**b**) transport study conducted with the apical buffer pH of 6.0 and the basal buffer of pH of 7.5. *Open bars* represent the apparent permeability from basal-to-apical direction, and *closed bars* represent the apparent permeability from apical-to-basal direction. The data are presented as the permeability*10^5, as mean ± SD, $n = 3$. This figure presents the data of one representative study, out of a total of three to four independent studies. Student's t-test was used to detect statistical significance, *$P < 0.05$.

DISCUSSION

GHB is a four-carbon chain monocarboxylic acid with a terminal hydroxyl group, which has been studied for its central nervous effects as a neurotransmitter and modulator (3). The clearance of GHB is believed to be mainly by metabolism through the Krebs-cycle intermediate (10,11). Our recent study on the renal clearance of GHB in rats

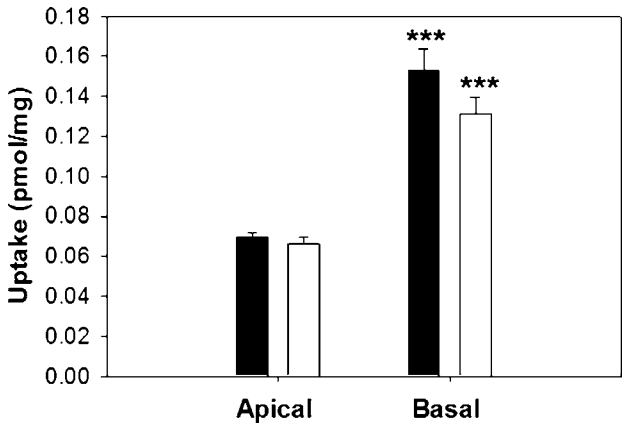


Fig. 8. Cellular uptake of GHB by HK-2 cells at 10 min during the directional flux studies. Basal represents adding GHB to the basal chamber, and apical represents adding GHB to the apical chamber. *Closed bars* represent the cellular accumulation when the buffer pH was 6.0, and *open bars* represent the cellular accumulation when the buffer pH was 7.5. The cellular accumulation, when dosing GHB in the basal chamber at pH 6.0, was significantly higher than that following dosing to the same chamber at pH 7.5 ($p < 0.05$, t-test). Data are presented as mean ± SD, $n = 3$, and this is one representative study of three independent studies. One-way ANOVA, followed by a Dunnett's test was used to detect statistical significance, compared with the uptake of GHB by HK-2 cells when dosing to the apical chamber at pH 7.5. *** $P < 0.001$.

demonstrated the importance of renal reabsorption of GHB (17). The renal handling of this compound, however, has not been well characterized. In this study, we studied GHB transport in human kidney cells and determined the transporters involved in GHB renal transport. Previous reports also demonstrated the presence of mRNA and protein of MCT1, 2 and 4 in mammalian kidney tissues (21,36,37). HK-2 cells retain the functional characteristics of proximal tubular epithelium of human kidney, and exhibit similar MCT expression as human kidney cortex tissues (33). We also demonstrated MCT1 protein to be present mainly on the basal membrane of polarized HK-2 monolayers (33). In this study, using a MCT substrate L-lactate, we demonstrated the functionality of MCT protein in HK-2 cells.

The driving force for GHB and L-lactate uptake in HK-2 cells was the pH gradient. The sodium gradient also affected the uptake of L-lactate and GHB at pH 7.5, but not at pH 6.0, and the uptake could be inhibited to the same level in the presence of CHC at both pH 6.0 and pH 7.5. Since it is known that CHC cannot inhibit sodium-dependent monocarboxylate transporter transport activity (38), this result suggested that sodium might not represent a direct transport driving force, and the effect might be indirect and mediated through other transporters such as Na/H antiporter. The similarity of the kinetic parameters of L-lactate and GHB uptake in HK-2 cells, and the similarity of the inhibitory effects of CHC and phloretin on L-lactate and GHB uptake, suggest that L-lactate and GHB may share common transporters in HK-2 cells. CHC exhibited competitive inhibition of GHB uptake; this is consistent with the mechanism of its inhibition of MCT-mediated L-lactate transport (19). The K_m of L-lactate uptake in HK-2 cells is similar to the literature value of MCT1-mediated uptake of L-lactate (19,39), and the K_m of GHB uptake in HK-2 cells is similar to the value determined in Ehrlich-lettre tumor cells (7.7 mM), where MCT1 is suggested to be the major transporter (39). The K_m of MCT4-mediated transport of GHB is estimated to be over 500 mM (40), which suggests that MCT4 may not be an important transporter in HK-2 cells. Although MCT3 has been shown to have a similar K_m value for L-lactate as reported for the MCT1, the MCT3-mediated transport of L-lactate is not inhibited by the MCT inhibitors CHC and phloretin (41). In this investigation, we observed more than 90% of inhibition on GHB uptake in HK-2 cells by CHC at 5 mM, so the extensive involvement of MCT3 in the transport of GHB is unlikely. pCMB is not an effective inhibitor for MCT2-mediated transport of L-lactate (19, 42); therefore, the fact that pCMB could inhibit GHB uptake to the same extent as CHC suggests that MCT2 may also not be an important transporter involved in GHB transport in HK-2 cells. In support of this finding, the uptake of GHB in HK-2 cells treated with siRNA for MCT1 was inhibited by 70%, while the uptake of GHB in HK-2 cells treated with the siRNA for MCT2 was only inhibited by 17%, and there was little effect on GHB uptake in HK-2 cells treated with the siRNA for MCT4; in all cases, the corresponding protein expressions were very low or negligible. When HK-2 cells were treated with the combination of siRNA for MCT1 and MCT2, or the combination of siRNA for MCT1 and MCT4, the uptake of GHB was decreased to a similar extent as observed with the siRNA for MCT1 alone.

The low pH-stimulated and CHC-inhibitable directional flux of GHB across HK-2 monolayers suggests the involvement of MCT1. The apparent permeability of GHB across HK-2 cell monolayers is composed of two separate routes: one is the transcellular route and the other is the paracellular route. The paracellular pathway will not be affected by buffer pH, but the transcellular pathway is affected due to the presence of MCT on HK-2 cell membranes. MCT1 is a tertiary active transporter, and the transport direction will be determined by the pH gradient. Basal localized MCT1 protein aided the flux of GHB from apical-to-basal when the pH gradient was from apical to basal, as demonstrated in Table II. The physiological pH gradient is usually low pH to high pH from lumen to blood, so the directional flux of GHB will be from lumen to blood, which represents active reabsorption. MCT1 served as primarily a facilitative transporter at pH 7.5 in the absence of a pH gradient, and no directional flux was observed. However, if the pH gradient was from basal-to-apical, MCT1 would increase the flux of GHB from basal-to-apical. The disappearance of this directional flux in the presence of CHC further suggested the importance of the role of MCT1 in the transport of GHB across HK-2 cell monolayers. A fraction of the transcellular transport of GHB is mediated by non-transporter mediated process, since GHB is also transported by a diffusion process in our cellular uptake studies, and this provides for the cellular accumulation of GHB in the presence of the MCT inhibitor CHC. Both the paracellular transport of GHB and the diffusion of GHB across HK-2 cell monolayers are responsible for the permeability of GHB in the presence of CHC. The possibility remains that an additional transporter on the apical membrane of HK-2 cells may aid in the directional flux of GHB. However, our cellular accumulation studies did not support this model, since the uptake of GHB by HK-2 cells after dosing in the apical chamber was always lower than that after dosing on the basal side: however, this does not rule out the possibility of other transporters.

Both pH- and sodium-dependent transport of GHB have been demonstrated in rat kidney brush-border membrane vesicles, while only the pH-dependent transport of GHB is present in basolateral membrane vesicles (43). In this study, we did not detect significant sodium-dependent transport of GHB in HK-2 cells, although a sodium-dependent monocarboxylate transporter has been demonstrated in human kidney (44). Although generally the apical transporter(s) are rate-limiting, the basal transporter(s) can also be critical to the overall transport. If the transport of GHB by MCT1 on the basal membrane is inhibited, GHB will accumulate in the tubular cells, and the reabsorption of GHB from tubular fluids will be decreased. Based on our current data, we were not able to conclude that MCT1 was the rate-limiting transporter for GHB transport, but we can conclude that MCT1 is an important basal transporter for GHB transport in human kidney cells. The sodium-dependent transporter(s) may also play a critical role in the reabsorption of GHB, but this remains to be investigated.

To our knowledge, this study represents the first study to demonstrate the transporter-mediated transport of GHB in human kidney cells. In summary, (1) MCT1, 2, and 4 are expressed in HK-2 cells; (2) L-lactate and GHB share a common transporter in HK-2 cells that is pH- and concen-

tration-dependent and can be inhibited by MCT substrates and inhibitors; and (3) MCT1 represents an important transporter of GHB transport in HK-2 cells.

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