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Monocarboxylate Transporter (MCT)-mediated transport of γ-hydroxybutyric acid in human intestinal Caco-2 cells

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Abbreviations: CHC, α-cyano-4-hydroxycinnamate; GHB, γ-hydroxybutyric acid; MCT, monocarboxylate transporter; pCMBS, p-chloromercuribenzenesulphonic acid; TEA, tetraethylammonium chloride;

ABSTRACT

The objectives of this study were to determine mRNA expression of MCT transporters, 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 were best described by a Michaelis-Menten equation with passive diffusion; GHB (K_m, 17.6 ± 10.5 mM; V_{max}, 17.3 ± 11.7 nmol/min/mg; P, 0.38 ± 0.15 μl/min/mg) and D-lactate (K_m, 6.0 ± 2.9 mM; V_{max}, 35.0 ± 18.4 nmol/min/mg; P, 1.3 ± 0.6 μl/min/mg). The uptake of GHB and D-lactate were 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 TEA. 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 the basolateral to apical direction. These findings confirm the presence of MCTs 1-4 in Caco-2 cells and demonstrate GHB and D-lactate transport characteristics consistent with proton-dependent MCT-mediated transport.

INTRODUCTION

Monocarboxylic acid transporters (MCTs), members of SLC16A family, are proton-linked transporters that play a crucial role in cellular metabolism. To date fourteen 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 (MCT 1-4) characterized as proton-dependent monocarboxylate transporters (Halestrap and Meredith, 2004; Bonen et al., 2006).

MCT 1 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 pre-translational 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 Felmlee, 2008). However, the specific isoforms vary in their substrate specificity and affinity, as well as 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 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-chloromercuribenzenesulphonic acid (pCMBS), a known MCT1 inhibitor (Halestrap and Meredith, 2004). In contrast to MCT2, MCT4 demonstrates lower affinities for monocarboxylates and is sensitive to pCMBS-mediated inhibition (Morris and Felmlee, 2008). MCT1 and MCT4 require a different ancillary protein as compared to 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 MCT substrates, D-lactate and GHB, a drug of abuse. Clinically, GHB is approved for the treatment of narcoleptic patients with cataplexy and marketed under the brand name of Xyrem® (Scharf et al., 1998), and for the treatment of alcohol withdrawal syndrome (Poldrugo and Addolorato, 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 role 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 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 that 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 (Hadjiagapiou 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.

METHODS

Chemical and Reagents

Minimum Essential Medium (MEM), Antibiotic-Antimycotic Solution (100x), fetal bovine serum and Hank's balanced salt solution (HBSS) were obtained from Gibco BRL (Buffalo, NY, USA). Sodium GHB, lithium D-lactate, α-cyano-4-hydroxycinnamate (CHC), tetraethylammonium chloride (TEA), HEPES, and 2-(N-morpholino)ethanesulfonic acid (MES) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ^{3}H-GHB (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA) and ^{3}H-D-lactate (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Biodegradable counting scintillate was obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Caco-2 cells were grown in T-75 cm^2 culture flasks with fresh culture medium replaced every other day. The culture medium consisted of MEM culture medium supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic solution (Gibco BRL, Buffalo, NY). All cells were incubated at 37^0C in a humidified atmosphere with 5 % CO_2/ 95 % air. Confluent Caco-2 cells were subcultured using 0.25% trypsin-EDTA in a ratio of 1:3 for T-75 cm^2 culture flasks or 1:12 for 35mm^2 culture dishes or Transwell inserts. For all the uptake studies, Caco-2 cells were seeded on 35 mm^2 culture dishes and used for experiments 2 days post-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 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 MCT 1-4 was performed as previously described (Wang et al., 2006a; Wang et al., 2006b). Briefly, 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 μg total RNA by reverse transcriptase using oligo dT primers and a Brilliant™ Two step QRT-PCR core reagent kit (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 200μM of each dNTP, 0.2 μM of each forward and reverse primer and 0.5U/reaction TAQ DNA polymerase (Fisher Scientific, Suwanee, GA) in 1X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3 (at 25 °C), 15 mM magnesium) through 40 cycles of 94 ^{o}C 1 minute, 60 ^{o}C 1 minute, 72 ^{o}C 1 minute. 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 35mm^2 culture dishes for 2 days were incubated with assay buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 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 pH 7.5. For sodium-dependent studies, cells were washed with assay buffer containing sodium (137 mM NaCl, 5.4 mM KCl, 2.8 mM

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$CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.5) or N-methyl-d-glucamine (137 mM N-methyl-d-glucamine, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.5). Uptake was initiated by the addition of 1ml 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 $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES, pH 7.5). One milliliter of lysis buffer containing 0.3N NaOH and 1 % SDS was added to solubilize the cells, and the radioactivity present in 200 µl aliquots was determined by liquid scintillation counting (1900 CA, Tri-Carb Liquid Scintillation Analyzer, Packard). α-Cyano-4-hydroxycinnimate (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 the BCA assay kit following the manufacturers' instructions.

GHB and D-lactate uptake kinetics were evaluated using simple Michaelis-Menton (Equation 1) or atypical kinetics (including multiple transporters and/or a diffusional component) (Equations 2-4) following initial Eadie-Hofstee plot evaluation. Transport kinetic parameters were evaluated by nonlinear regression analysis (WinNonLin 5.1 Pharsight Corp., Cary, NC). The model of best fit was selected based on a visual inspection of fits and a comparison of Akaike Information Criterion (AIC) values.

$$v = \frac{V_{\max} * C}{K_m + C} \qquad \text{[Equation 1]}$$

$$v = \frac{V_{\max} * C}{K_m + C} + P * C \qquad \text{[Equation 2]}$$

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$$v = \frac{V_{\max 1} * C}{K_{m1} + C} + \frac{V_{\max 2} * C}{K_{m2} + C} \qquad \text{[Equation 3]}$$

$$v = \frac{V_{\max 1} * C}{K_{m1} + C} + \frac{V_{\max 2} * C}{K_{m2} + C} + P * C \qquad \text{[Equation 4]}$$

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 ^{3}H-GHB or ^{3}H-D-lactate. The studies were performed as described by Zhang and Morris (2003). Briefly, Caco-2 cells on Transwell permeable supports with transepithelial electrical resistance (TEER) values higher than 500 Ω·cm^2 were washed with transport buffer (137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaC1_2$, 1.2 mM $MgCl_2 \cdot 6H_20$, 5% glucose, 10 mM MES for pH 6.0 buffer or 10 mM HEPES for pH 7.5 buffer) three times at 37^{o}C. Transport buffer containing radiolabeled substrate was added to the apical (AP, 1.5 ml) or basolateral (BL, 2.6ml) chamber while non-radiolabeled transport buffer was added to the opposite chamber. Samples were incubated at 37^{o}C and 100 μl aliquots were taken from the receiver side at 30, 60 and 90 minutes for liquid scintillation counting (Wang et al., 2006b).

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

$$Papp = \frac{\Delta Q}{\Delta t} * \frac{1}{C_o * A} \qquad \text{[Equation 5]}$$

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 Inc., San Diego, CA). Significant differences between means were determined by one-way ANOVA followed by a Dunnett's post hoc test or a two-way ANOVA with a Bonferroni post hoc test. $P < 0.05$ was considered as statistically significant.

RESULTS

MCT Expression in Caco-2 cells

In agreement with previous studies (Hadjiagapiou 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 and Figure 1). Our study also demonstrated mRNA expression of MCT2 in Caco-2 which was not observed or assessed in previous studies.

[^{3}H]GHB and D-lactate Uptake Studies

Preliminary studies demonstrated GHB and D-lactate uptake was linear up to 10 minutes (data not shown), and an incubation time of 5 minutes 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 ^{3}H-GHB or ^{3}H-D-lactate at pH values ranging from pH 5.5 to pH 7.5 (Figure 2A & B). The uptake rates of GHB and D-lactate increased with decreasing pH with significantly higher uptake rates observed at pH 5.5, pH 6.0 and pH 6.5 (D-lactate only) when compared to 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 as this pH is more clinically relevant than a lower pH value of 5.5, as well as to minimize the changes in the ionization state of GHB which could possibly influence diffusion.

In contrast, incubation of GHB and D-lactate with and without sodium resulted in no difference in the rate of uptake (Figure 2C & 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 mM to 80 mM for GHB and 0.05 mM to 30 mM for D-lactate) with uptake determined at an extracellular pH of 6.0. The uptake of GHB and D-lactate were described by a Michaelis-Menten equation with passive diffusion (Equation 2); GHB (Km, 17.6 ± 10.5 mM; Vmax, 17.3 ± 11.7 nmol/min/mg; P, 0.38 ± 0.15 µl/min/mg) and D-lactate (Km, 6.0 ± 2.9 mM; Vmax, 35.0 ± 18.4 nmol/min/mg; P, 1.3 µl/min/mg) (Figure 3). Eadie-Hofstee plots of GHB and D-lactate uptake suggested the involvement of two transport processes (data not shown); however, the data was not well described by Equation 3 suggesting that two saturable transport processes may not be involved in uptake or an insufficient concentration range was not assessed.

Influence of MCT Inhibitors

The effect of inhibitors on the transport of GHB and D-lactate were 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 (Figure 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; however, the specific isoform(s) involved in transport remains to be determined as CHC is a non-specific MCT inhibitor (Morris and Felmlee, 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 statistical significant difference when pH 7.4 buffers were added to both chambers (Figure 5). However, the AP-to-BL transport of both GHB and D-lactate were 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-BL direction at 30 and 60 minutes (Figure 6A & 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 (Figure 6C &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 γ-aminobutyric acid (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 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 previously reported (Hadjiagapiou et al., 2000; Gill et al., 2005; Englund et al., 2006), with the expression of MCT1 found to be high relative to 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 were demonstrated to occur in a concentration- and proton gradient-dependent manner suggesting the involvement of MCTs. We observed a 3.6- and 3.0-fold increase 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 sodium-coupled monocarboxylate transporters (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 likely 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 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 is still controversial.

The involvement of MCTs in the uptake of GHB and D-lactate was further confirmed by the significant decrease in uptake following co-incubation with a known MCT inhibitor CHC and MCT substrates. These results are consistent the observed inhibition of MCT-mediated 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 non-specific 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, while 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-Menton equation with a diffusional clearance term (Equation 2). Previous studies conducted in our laboratory observed 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-Menton equation in HK-2 cells (Wang et al., 2006b; Wang et al., 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 was not well described by Equation 3. On the other hand, the data was 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 likely present in the highest amounts and uptake may be predominantly due to MCT1. Additionally, even though the data is best fit to a single Michaelis-Menton 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 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 Km 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, since 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 of 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 MCT 1 - 4 mRNA in Caco-2 cells was detected. The proton-dependent nature of uptake and the inhibition by MCT substrates and the classical 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; Wang et al., 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 transporter(s) in the intestinal transport of moncarboxylates.

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FOOTNOTES

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FIGURE LEGENDS

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

Figure 2: Driving forces of GHB uptake in Caco-2 cells. Influence of pH on (A) GHB (0.1 mM) and (B) D-lactate (0.1 mM) uptake. Data are expressed as a percentage of control (pH 7.5). Influence of sodium on (C) GHB (0.1 mM) and (D) D-lactate (0.1 mM) uptake. Data are expressed as percentage of control (without sodium). Results are presented as mean ± SEM for three independent experiments performed in triplicate. *$P < 0.05$ as compared to control.

Figure 3: Concentration-dependent uptake of (A) GHB and (B) D-lactate in Caco-2 cells. Experiments were performed at pH 6.0 with uptake determined at 5 minutes. Data are expressed as mean ± SEM of two independent experiments performed in triplicate. The line represents the model prediction.

Figure 4: Effects of MCT inhibitors on (A) GHB (0.1 mM) and (B) D-lactate (0.1 mM) 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 (5mM). Results are presented as mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ as compared to control.

Figure 5: Bidirectional transport of (A) ^{3}H-GHB (conc) and (B) ^{3}H-D-lactate across Caco-2 cell monolayers in the absence of a pH gradient. (□) Permeability from AP to BL and (■)

Permeability from BL to AP. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

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

TABLES

Table 1: Primers used for RT-PCR analysis.

Isoform	Accession Number	Direction	Sequences	Product
MCT1	NM_003051	Forward	5'-TGGATGGAGAGGAAGCTTTCTAAT-3'	151
		Reverse	5'-CACACCAGATTTTCCAGCTTTC-3'	
MCT2	NM_004731	Forward	5'-GCCCACTGGCACAGGACTA-3'	251
		Reverse	5'-CACAATAGCCCCACAGGACAT-3'	
MCT3	NM_013356	Forward	5'-GGATGCGTTGAAGAACTATGAGATC-3'	213
		Reverse	5'-CCGGGTTCCTCTGCAACA-3'	
MCT4	NM_004207	Forward	5'-CACGGCATCGTCACCAACT-3'	200
		Reverse	5'-ACAGCCTGGATAGCAACGTACAT-3'	

FIG 1.

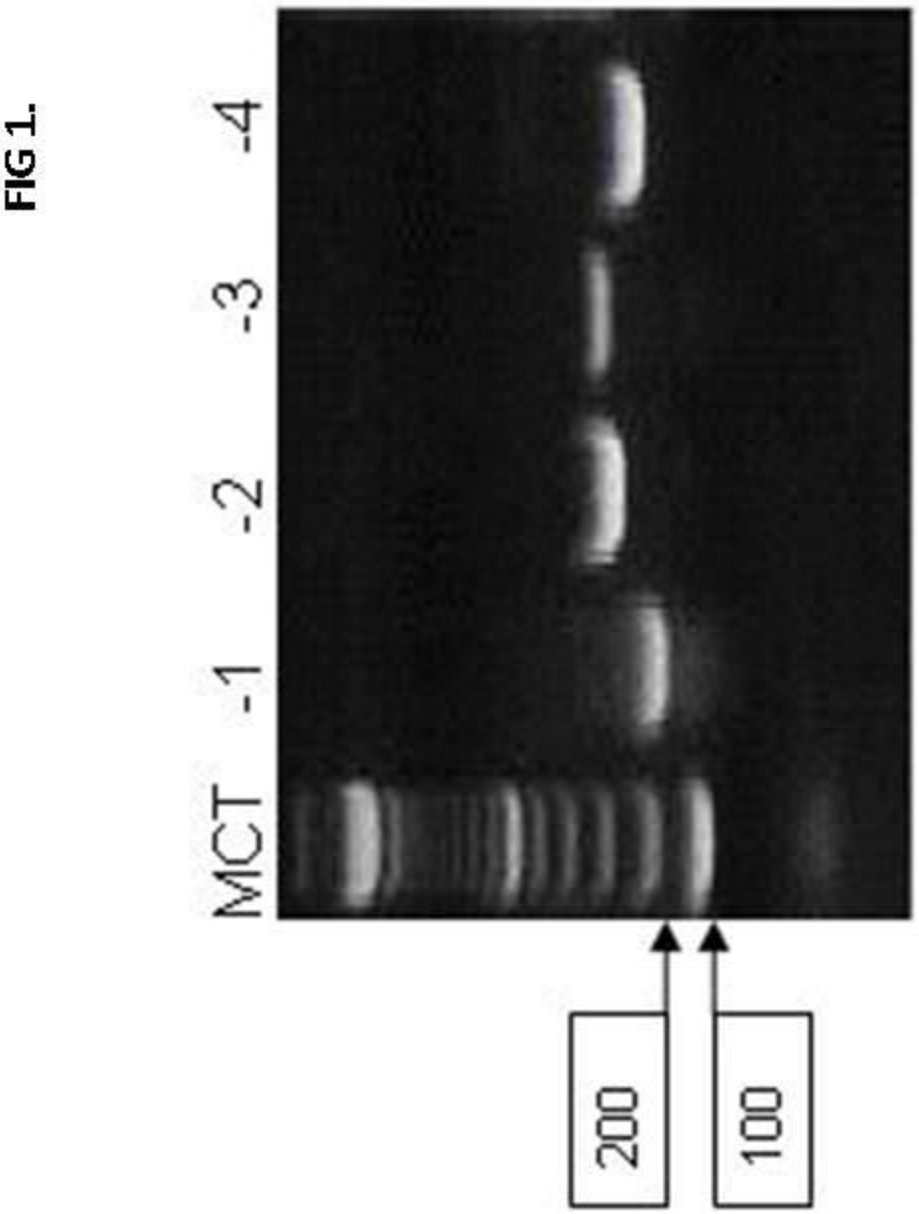


FIG 2.

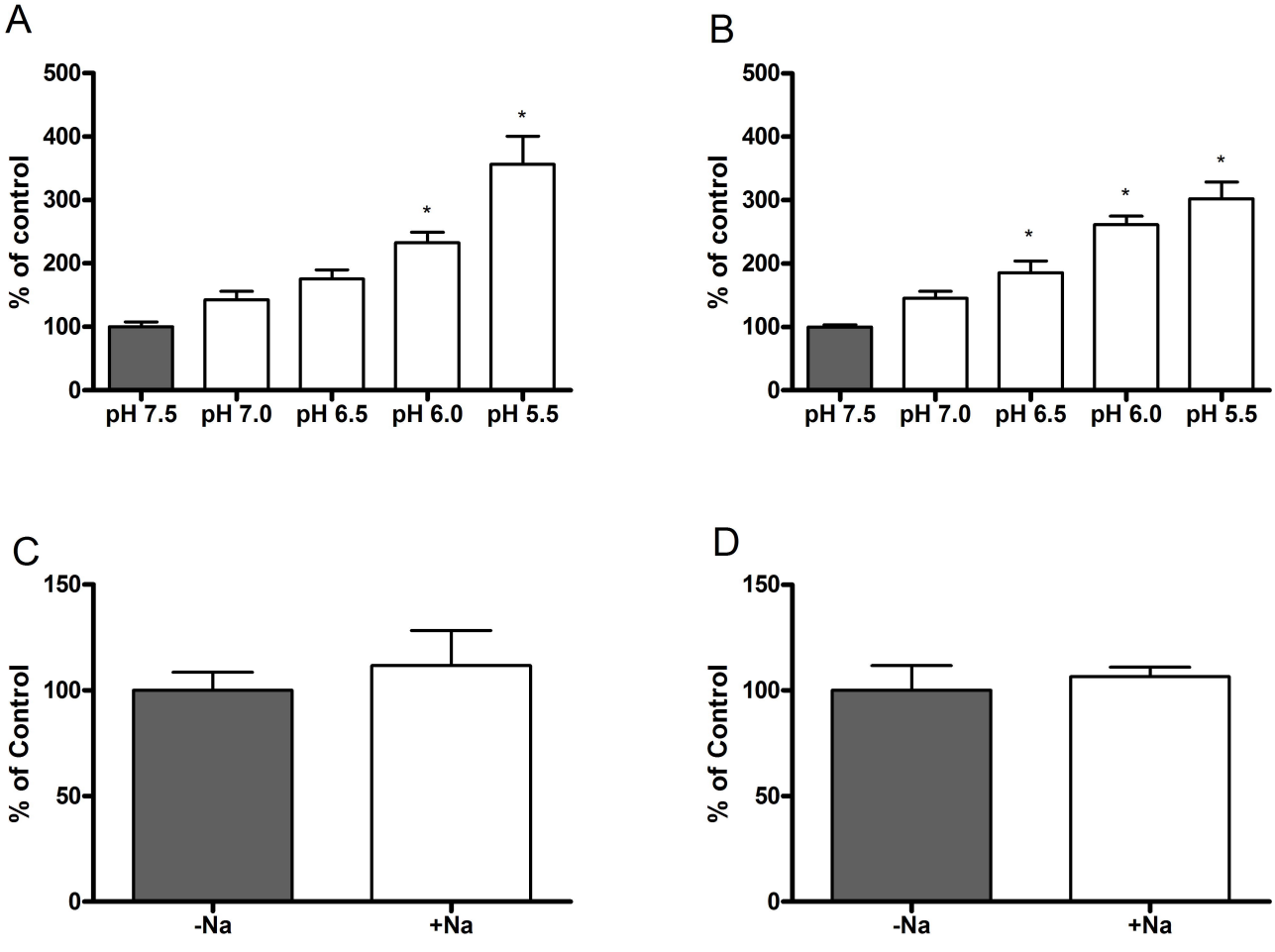

A
500
400
300
200
100
0
% of control
*
*
pH 7.5
pH 7.0
pH 6.5
pH 6.0
pH 5.5
B
500
400
300
200
100
0
% of control
*
*
*
pH 7.5
pH 7.0
pH 6.5
pH 6.0
pH 5.5
C
150
100
50
0
% of Control
-Na
+Na
D
150
100
50
0
% of Control
-Na
+Na

FIG 3.

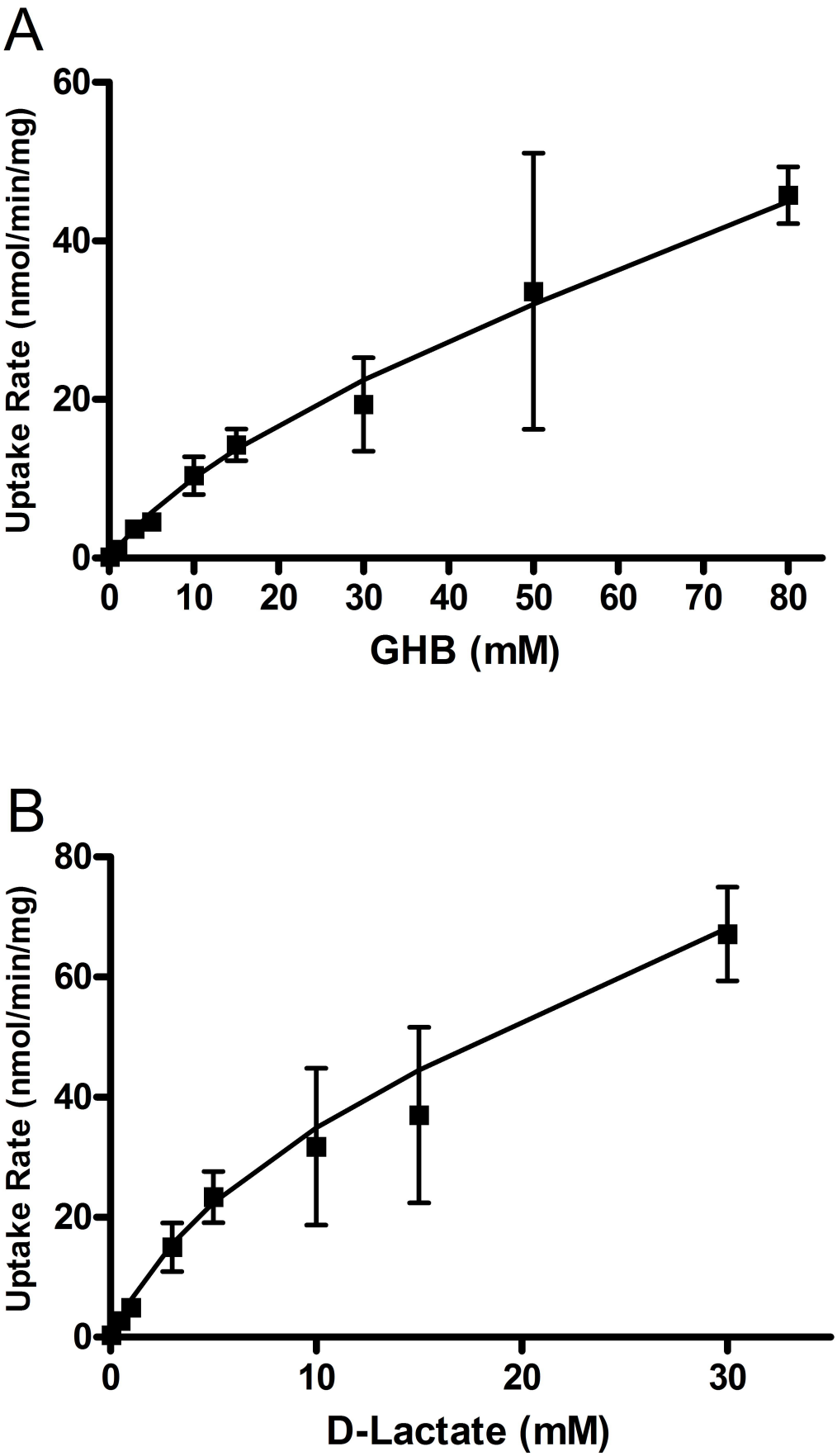
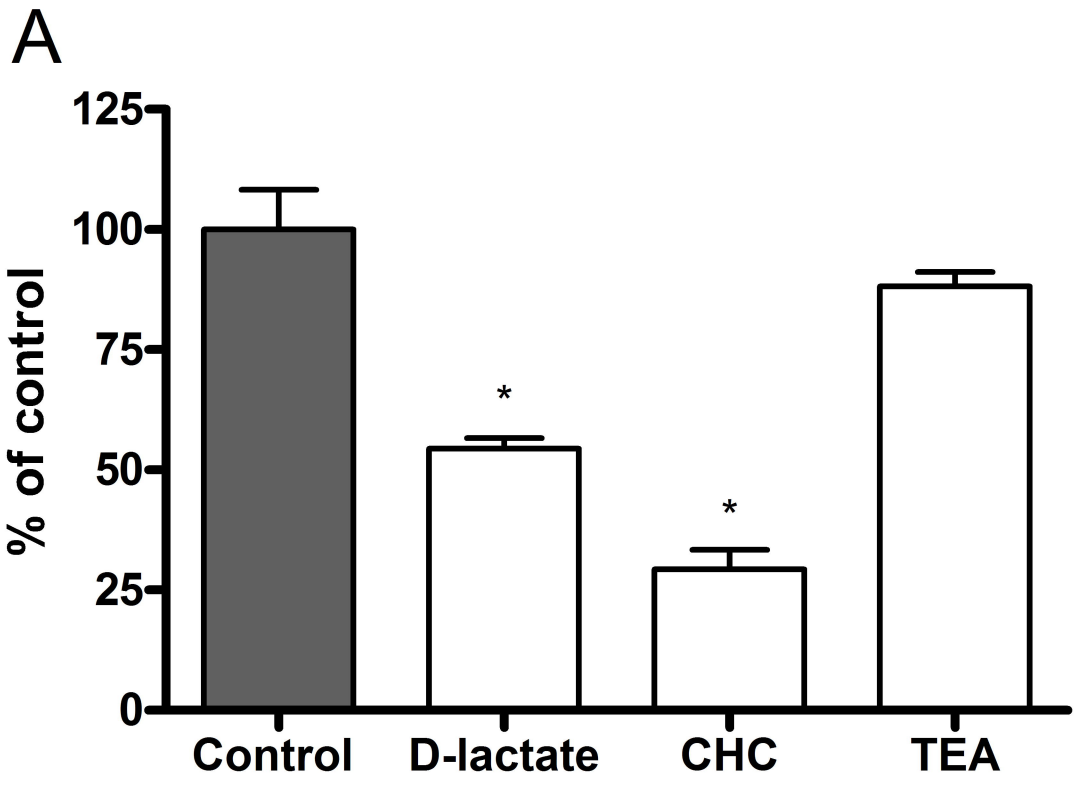

A
Uptake Rate (nmol/min/mg)
60
40
20
0
0
10
20
30
40
50
60
70
80
GHB (mM)
B
Uptake Rate (nmol/min/mg)
80
60
40
20
0
0
10
20
30
D-Lactate (mM)

FIG 4.


A
125
100
75
50
25
0
% of control
*
*
Control
D-lactate
CHC
TEA

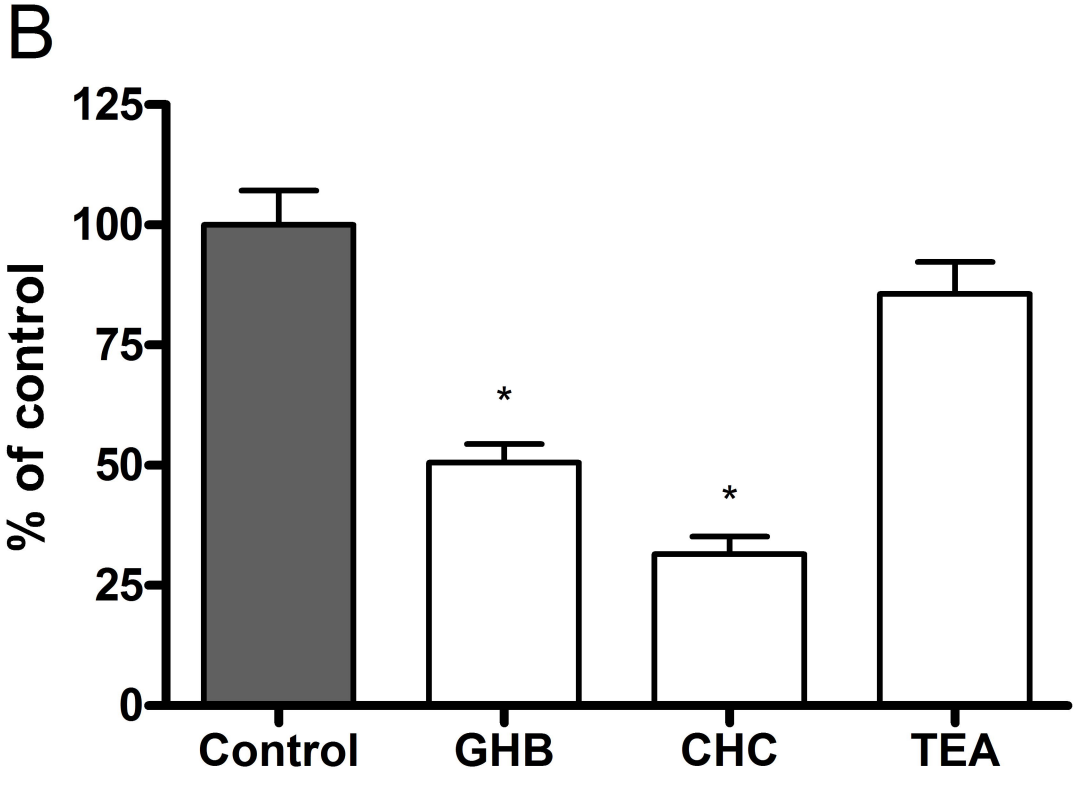
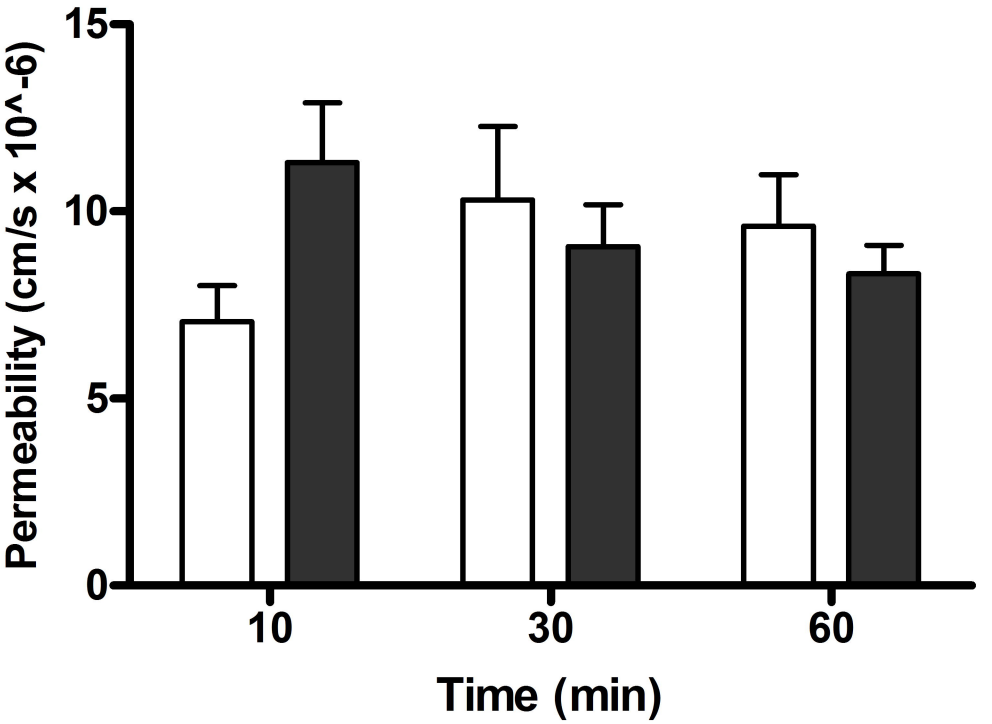

B
125
100
75
50
25
0
% of control
*
*
Control
GHB
CHC
TEA

FIG 5.

A


Permeability (cm/s x 10^-6)
15
10
5
0
10
30
60
Time (min)

B

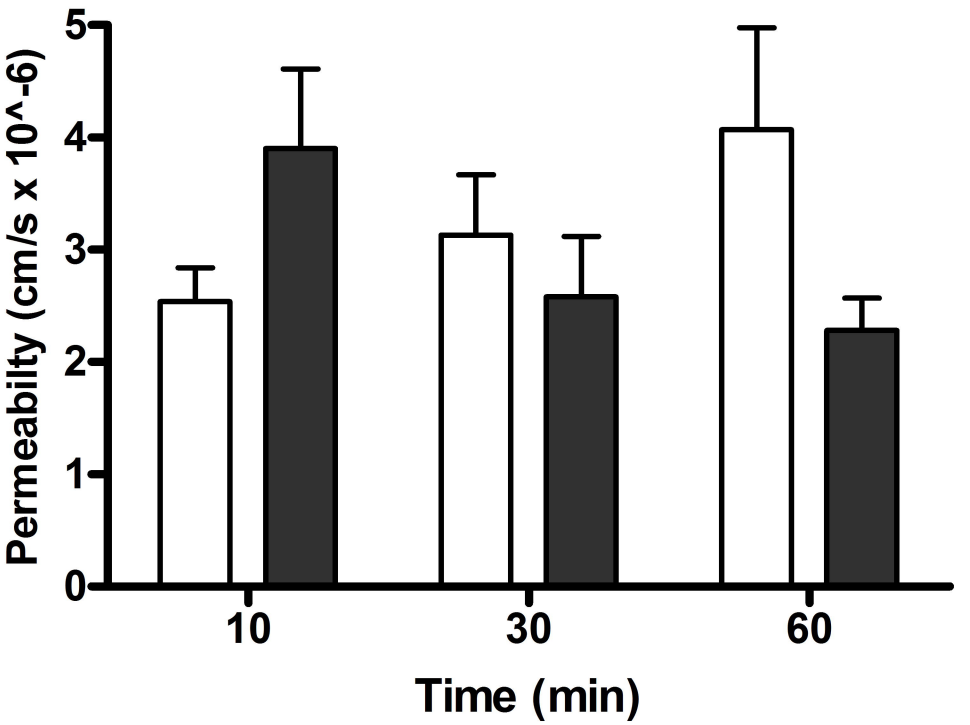
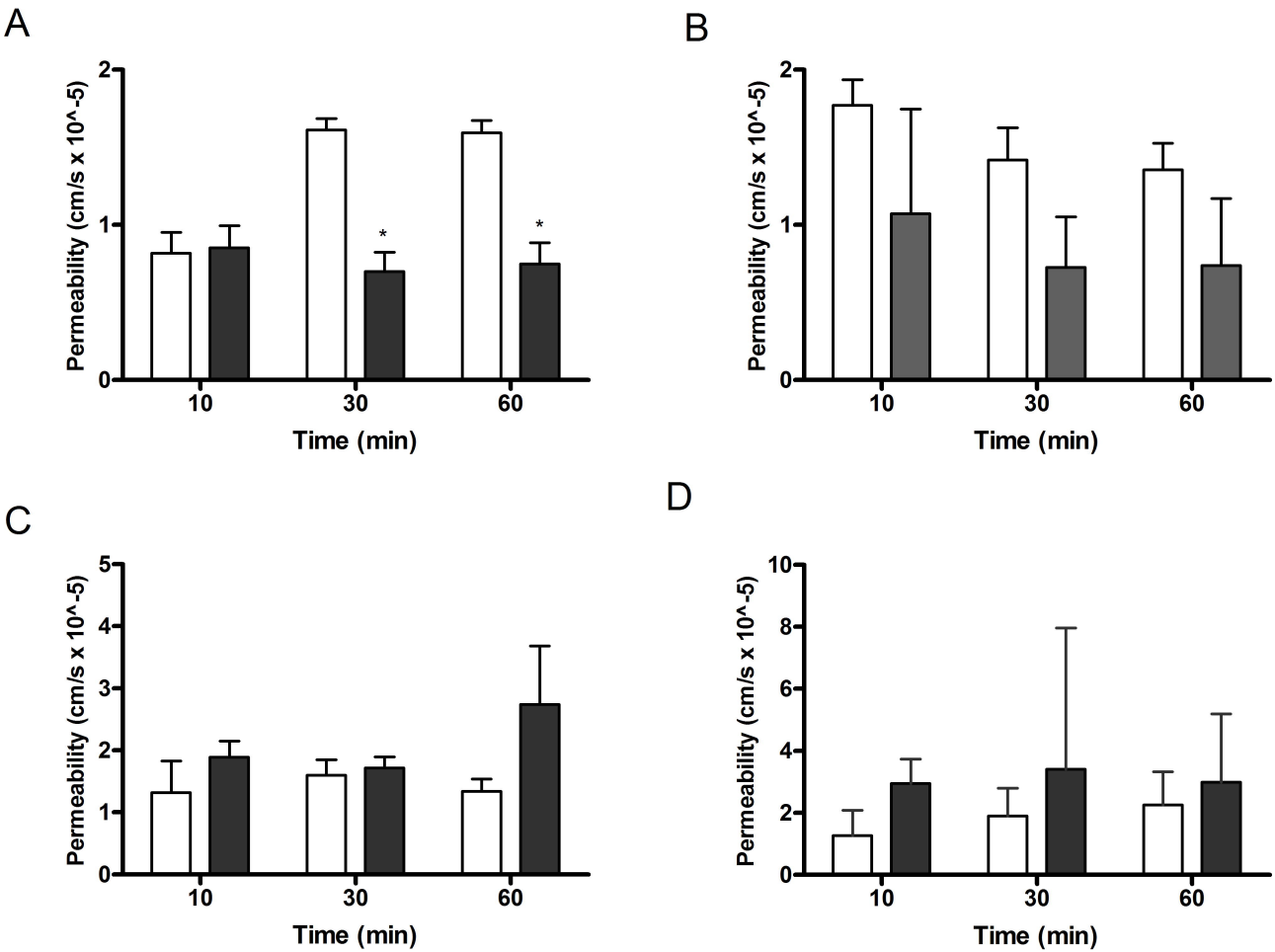

Permeabilty (cm/s x 10^-6)
5
4
3
2
1
0
10
30
60
Time (min)

FIG 6.


A
Permeability (cm/s x 10^-5)
2
1
0
10
30
60
Time (min)
*
*
B
Permeability (cm/s x 10^-5)
2
1
0
10
30
60
Time (min)
C
Permeability (cm/s x 10^-5)
5
4
3
2
1
0
10
30
60
Time (min)
D
Permeability (cm/s x 10^-5)
10
8
6
4
2
0
10
30
60
Time (min)