Structural Characterization of Substrates for the Anion Exchange Transporter in Caco-2 Cells[||]

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Received January 12, 1999. Final revised manuscript received May 27, 1999.
Accepted for publication August 10, 1999.

Abstract □ The present study was conducted to characterize the structural specificity of an anion exchange transporter in intestinal epithelial cells. The transport of carboxylic acids with hydroxyl group(s) at the 2, 3, 4, and/or 5 positions with respect to carboxylate was examined by using Caco-2 cells in the presence of bicarbonate ions on the basolateral side to enhance the activity of the anion-exchange transporter. In the presence of the bicarbonate ion gradient, transport of L-lactic acid consisted of a saturable process and a nonsaturable process as judged from the Eadie–Hofstee plot. The transport of L-lactic acid at 1 μM was reduced by sodium azide, dinitrophenol, and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS). It was also reduced by 2-, 4-, and 5-hydroxycarboxylic acids such as hydroxyacetic acid, 4-hydroxybutyric acid, and 5-hydroxydecanoic acid, but not by 3-hydroxycarboxylic acids such as 3-hydroxypropionic acid and 3-hydroxybutyric acid. Transport of both 2- and 4-hydroxybutyric acids involved saturable and nonsaturable processes, whereas that of 3-hydroxybutyric acid was nonsaturable and was not inhibited by DIDS. These results indicate that 3-hydroxycarboxylic acids might not be substrates for this anion exchange transporter in intestinal epithelial cells, suggesting that the position of hydroxylation is significant for molecular recognition by the transporter.

Introduction

We have demonstrated that at least two types of carrier-mediated transport mechanisms, energized by an inward-directed proton gradient (proton cotransport) and/or an outward-directed bicarbonate gradient (anion-exchange transport), are involved in the intestinal absorption of monocarboxylic acids such as acetic acid, L- and D-lactic acids, nicotinic acid, benzoic acid, and salicylic acid, by means of studies using rabbit and rat jejunal brush-border membrane vesicles (BBMVs), a human carcinoma cell line, Caco-2, and gene products expressed in *Xenopus laevis* oocytes and in mammalian cells.[1−13] These carrier-mediated transport processes are likely to predominate over passive diffusion according to the pH-partition hypothesis[14] in the absorption of various monocarboxylic acids across the intestinal membrane.

Previously, we have demonstrated that pravastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, and (*R*)-mevalonic acid, an intermediate in terpenoid and cholesterol biosynthesis, are transported across intestinal epithelial cells via a proton-cotransport mechanism. However, these compounds are not transported by an anion-exchange transport mechanism in intestinal BBMVs or in isolated intestinal tissues mounted in Ussing-type chambers.[11] Pravastatin and (*R*)-mevalonic acid have a structural similarity in terms of the hydroxyl groups at the 3 and 5 positions with respect to the carboxylate, so such structural characteristics might determine their transport across the intestinal epithelial cells via the anion-exchange transport mechanism. The present study was intended to characterize the common structure of monocarboxylic acids which is required for transport via the anion-exchange transporter in intestinal epithelial cells, by examining the transcellular transport of substituted monocarboxylic acids, especially those with a hydroxyl group at the 2, 3, 4, or 5 position of the carboxylates.

Materials and Methods

Materials and Cell Culture—Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) and cultivated as described previously.[9] All cells used in this study were between passages 53 and 108. The cells were grown for 21 to 23 days on a polycarbonate membrane (Transwell: 11.2 mm in diameter and 3.0 μm pore size, effective area of 1 cm^2, Costar, Bedford, MA). L-[^{14}C]Lactic acid (5.55 GBq/mmol), [^{14}C]-2-hydroxybutyric acid (2.04 GBq/mmol), [^{14}C]-3-hydroxybutyric acid (2.05 GBq/mmol), [^{14}C]-4-hydroxybutyric acid (2.04 GBq/mmol), and (*R*)-[^{3}H]mevalonolactone (555 GBq/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Mevalonic acid was prepared by hydrolysis of the mevalonolactone according to the method reported previously.[11,15] [^{14}C]Salicylic acid (2.04 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). [^{14}C]-Benzoic acid (0.70 GBq/mmol) and [^{3}H]mannitol (1110 GBq/mmol) were purchased from New England Nuclear (Boston, MA). 5-Hydroxydecanoic acid was kindly supplied by Mochida Pharmaceutical Co., Ltd. (Tokyo). All other chemicals were of reagent grade or the highest purity commercially available.

Transcellular Transport Experiments—The conditions of each experiment are described in figure legends or table footnotes. A typical experiment on L-lactic acid transport was performed as follows. For the study of the transcellular transport across Caco-2, the cells grown on a polycarbonate membrane were washed twice with Hank's balanced salt solution (HBSS; 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 30 mM D-glucose, and 10 mM HEPES for pH 7.4 or 10 mM MES for pH 6.0), and the osmolarity was adjusted to 315 mOsm/kg.[9] To initiate transport, 1.5 mL of HBSS (pH 7.4, 37 °C) was put on the basolateral side (receiver side), and 0.5 mL of the test solution (pH 6.0 or pH 7.4, 37 °C) containing a radio-labeled compound was loaded on the apical side (donor side) of a cell insert. At 15, 30, 45, and 60 min after that, 0.5-mL

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|| Abbreviations: BBMVs, brush-border membrane vesicles; HBSS, Hank's balanced salt solution; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; DNP, 2,4-dinitrophenol; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; ^{1}H NMR, proton nuclear magnetic resonance.

10.1021/js9900093 CCC: $18.00
Published on Web 09/24/1999

aliquots of the solution were removed from the receiver side and replaced with an equal volume of fresh HBSS. In the case of the bicarbonate-gradient condition, modified HBSS (0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 111.7 mM NaCl, 0.385 mM Na_2HPO_4, 25 mM $NaHCO_3$, 30 mM D-glucose, and 10 mM HEPES, pH 7.4 with the osmolarity of 315 mOsm/kg, 37 °C), which contained a suitable amount of bicarbonate ion to maintain the bicarbonate gradient during the experiments, was used on the basolateral side. The amount of the radio-labeled compound transported by cells was estimated by radioactivity measurement and expressed as permeability (μL/mg protein), obtained by dividing the transported amount by the initial concentration in the donor compartment and correcting for the cellular protein amount. Each result represents the mean ±SEM of three experiments using the same cultivation of Caco-2 cells. Radioactivity was determined with a liquid scintillation counter (LS6000TA, Beckman, Fullerton, CA). Cellular protein was measured by the method of Lowry et al.[16] with bovine serum albumin as a standard.

Measurement of Physicochemical Properties—The partition coefficient of each compound between *n*-octanol and HBSS (pH 7.4), log $D_{7.4}$, was determined by means of the flask shaking method.[17] The proton nuclear magnetic resonance (^{1}H NMR; 300 MHz) spectra were obtained in dimethyl sulfoxide-d_6 using tetramethylsilane as an internal standard at room temperature.

Data Analysis—The permeability coefficient (μL/min/mg protein) was determined from the slope of the initial linear portion of the permeability (μL/mg protein) versus time (min) curves by linear regression analysis. A permeability coefficient of 1 μL/min/mg protein corresponded to 15.4×10^{-6} cm/s, since cellular protein averaged 0.922 mg/cm^2. To estimate the kinetic parameters for the saturable transport across Caco-2 monolayers, the transport rate (J) was fitted to one of the following equations, consisting of both saturable and nonsaturable-linear terms, by using a nonlinear least-squares regression analysis program (WinNonlin, SCI, Apex, NC),

$$J = J_{max} \times S/(K_t + S) + k_d \times S \quad (1)$$

or

$$J = J_{max,1} \times S/(K_{t,1} + S) + J_{max,2} \times S/(K_{t,2} + S) + k_d \times S \quad (2)$$

where J_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, K_t is the half-saturation concentration (Michaelis constant), subscript integers 1 and 2 indicate saturable processes of high and low affinity, respectively, and k_d is the first-order rate constant. Statistical analysis was performed by using Student's two-tailed t test. A difference between means was considered to be significant when the P-value was less than 0.05.

Results

pH and Bicarbonate Ion Dependence of L-Lactic Acid Transport—Figure 1 shows the time-courses of the permeability of L-[^{14}C]lactic acid and [^{3}H]mannitol from the apical to the basolateral side across a Caco-2 cell monolayer in the presence or absence of a proton or bicarbonate gradient, or both. The permeability of L-[^{14}C]lactic acid increased linearly with time after an initial lag period of a few minutes, as reported previously.[9] In the bicarbonate-free condition, the permeability coefficient of L-[^{14}C]lactic acid at the apical pH of 6.0 was 0.365 ± 0.020 μL/min/mg protein, being significantly higher than that (0.176 ± 0.010 μL/min/mg protein) observed at the apical pH of 7.4. In the presence of 25 mM bicarbonate ions on the basolateral side, the permeation rates of L-[^{14}C]lactic acid at the apical pH values of 6.0 and 7.4 were 1.32 and 1.33 times faster (0.482 ± 0.031 and 0.233 ± 0.010 μL/min/mg protein, respectively) than those observed in the absence of a bicarbonate ion gradient, respectively. Since the permeability coefficients of L-[^{14}C]lactic acid under all conditions examined were significantly higher than that (0.087 ± 0.001 μL/min/mg protein) of [^{3}H]mannitol, which reflects the paracellular permeability, L-lactic acid transport occurred mainly by transcellular permeation rather than through the paracellular pathway.

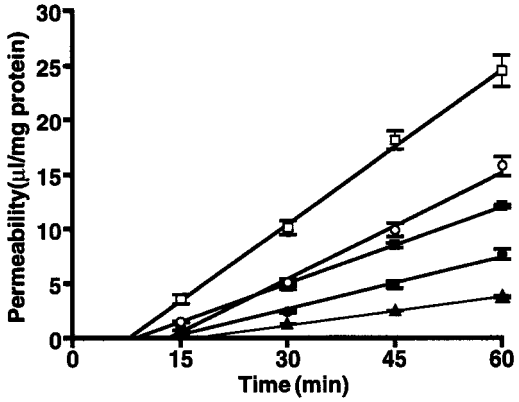

Figure 1—Time-courses of the transport of L-[^{14}C]lactic acid and [^{3}H]mannitol from the apical side to the basolateral side across Caco-2 cell monolayers. Permeability of L-[^{14}C]lactic acid (1 μM) was measured at 37 °C by incubating Caco-2 monolayers in HBSS buffer at an apical pH of 6.0 (□, ○), or 7.4 (●, ■) with a constant basolateral pH of 7.4, in the presence of 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM K_2HPO_4, 25 mM D-glucose and 10 mM HEPES for pH 7.4 or 10 mM MES for pH 6.0 (circles). When bicarbonate-containing HBSS buffer at a basolateral pH of 7.4 was used (squares), 111.7 mM NaCl and 25 mM $NaHCO_3$ were contained instead of 136.7 mM NaCl. [^{3}H]Mannitol (33 nM; ▲) transport was measured at 37 °C in Caco-2 monolayers in HBSS buffer at an apical pH of 7.4 and bicarbonate-containing HBSS buffer at a basolateral pH of 7.4. An aliquot was withdrawn from the basolateral side at each time indicated. Each point represents the mean ± SEM of three experiments using the same culture of Caco-2 cells.

Concentration Dependence of L-Lactic Acid Transport—Figure 2 illustrates the relationship between the initial transport rate of L-[^{14}C]lactic acid and the concentration in the medium (from 1 μM to 20 mM) under the two different conditions, namely at the apical pH values of 6.0 (panel A) and 7.4 (panel B), while the pH on the basolateral side was kept at 7.4 with 25 mM bicarbonate ions. The results indicate that the permeation rate of L-[^{14}C]lactic acid under both conditions involves both saturable and nonsaturable processes, as shown in the inset of Figure 2. When the data at the apical pH of 6.0 were analyzed by means of an Eadie–Hofstee plot, two different types of transport processes were observed, as illustrated in panel A. Kinetic analysis of the concentration-dependent permeation according to eq 2, gave $J_{max,1}$ and $K_{t,1}$ values of 0.098 ± 0.021 nmol/min/mg protein and 0.28 ± 0.10 mM, and $J_{max,2}$ and $K_{t,2}$ values of 1.46 ± 0.20 nmol/min/mg protein and 10.9 ± 2.0 mM, respectively. The k_d value was 0.056 ± 0.043 μL /min/mg protein (mean ± SD). At the apical pH of 7.4, the Eadie–Hofstee plot apparently shows a single saturable process. Kinetic analysis of L-lactic acid transport according to eq 1 gave J_{max}, K_t, and k_d values of 1.03 ± 0.36 nmol/min/mg protein, 8.26 ± 2.80 mM, and 0.056 ± 0.010 μL/min/mg protein, respectively. Accordingly, the transport of L-[^{14}C]lactic acid across Caco-2 cell monolayers seems to be bicarbonate-ion dependent. So, in subsequent experiments, transport studies were performed at apical and basolateral pH values of 7.4 in the presence of 25 mM bicarbonate ions on the basolateral side to magnify the relative activity of anion-exchange transport.

Inhibitory Effect on Monocarboxylate Transport—Table 1 shows the effects of the bicarbonate ion gradient, metabolic inhibitors, and monocarboxylates on the transport of L-[^{14}C]lactic acid, [^{3}H]mevalonic acid, [^{14}C]benzoic acid, [^{14}C]salicylic acid, and [^{3}H]mannitol. DNP (1 mM), an uncoupler of oxidative phosphorylation, sodium azide

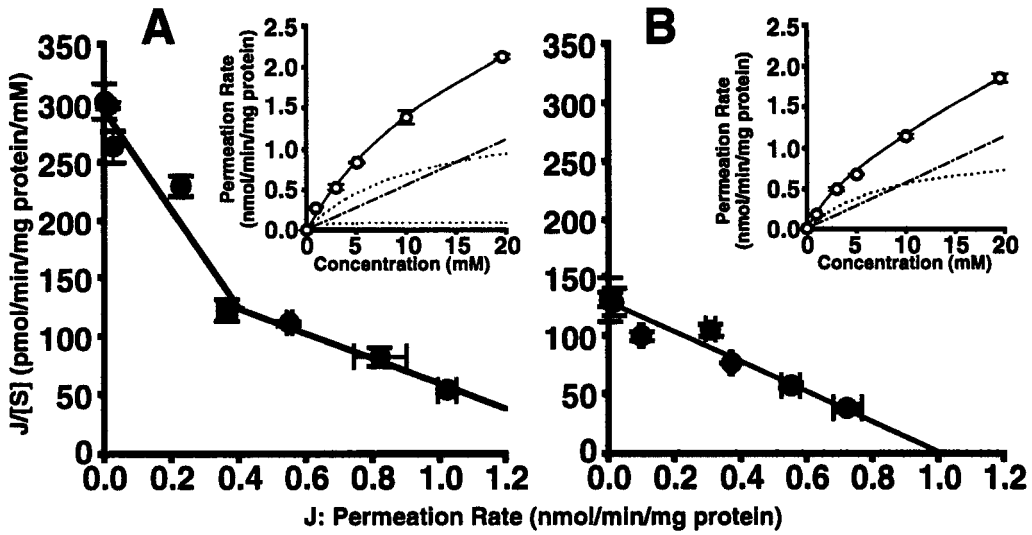

Figure 2—Concentration dependence of L-[^{14}C]lactic acid transport across Caco-2 cell monolayers at an apical pH of 6.0 (panel A) or 7.4 (panel B), at a constant basolateral pH of 7.4 with 25 mM bicarbonate. The concentrations of L-lactic acid used were 1, 10, and 100 μM and 1, 3, 5, 10, and 20 mM, which were adjusted by addition of the nonlabeled compound to L-[^{14}C]lactic acid (1 μM). The incubation conditions were identical to those described in the legend to Figure 1. The broken lines represent the contribution of the saturable (••••) and nonsaturable (–•–) components to the permeation, calculated from the kinetic parameters obtained as described in the results. Each point represents the mean ± SEM of three experiments using the same culture of Caco-2 cells.

Table 1—Effect of Various Compounds and Condition on Monocarboxylic Acid and Mannitol Transports[a]

condition	relative permeability (% of control)[b]				
	L-[^{14}C]lactic acid	(*R*)-[^{3}H]mevalonic acid	[^{14}C]benzoic acid	[^{14}C]salicylic acid	[^{3}H]mannitol
control	100.0 ± 3.8	100.0 ± 12.2	100.0 ± 0.7	100.0 ± 5.7	100.0 ± 1.8
bicarbonate free	75.4 ± 4.1*	88.2 ± 1.8	52.9 ± 1.3*	40.4 ± 1.6*	99.8 ± 3.4
+1 mM DNP	43.9 ± 2.2*				106.2 ± 2.9
+10 mM NaN_3	54.5 ± 6.0*				101.2 ± 3.9
+2 mM DIDS	32.4 ± 1.1*	97.4 ± 0.9	45.4 ± 0.5*	41.4 ± 1.9*	98.0 ± 6.4
+10 mM L-lactic acid	45.8 ± 3.2*				
+10 mM (*R*)-mevalonic acid	88.4 ± 5.8	86.8 ± 6.4			
+10 mM (*S*/*R*)-mevalonic acid	104.8 ± 6.0				
+10 mM benzoic acid	42.0 ± 6.1*		59.8 ± 1.3*		
+10 mM salicylic acid	47.6 ± 1.3*			57.9 ± 3.6*	

[a] Note: Transport of L-[^{14}C]lactic acid (1 μM), (*R*)-[^{3}H]mevalonic acid (50 nM), [^{14}C]benzoic acid (10 μM), [^{14}C]salicylic acid (10 μM), and [^{3}H]mannitol (33 nM) were measured at 37 °C for 60 min by incubating Caco-2 cells in HBSS buffer (apical pH 7.4, basolateral pH 7.4 with bicarbonate) in the presence of each inhibitor. The other incubation conditions were the same as described in the legend to Figure 1. [b] Each value represents the mean ± SEM of three experiments using the same cultivation process of Caco-2 cells and is expressed as percentage of the control. *Significantly different from the control value by Student's *t* test ($p < 0.05$).

(10 mM), a respiratory chain inhibitor, and DIDS (2 mM), an anion exchange inhibitor, significantly diminished the transport of L-[^{14}C]lactic acid. Salicylic acid and benzoic acid (10 mM) were inhibitory, whereas neither (*R*)- nor racemic mevalonic acid (10 mM) had any effect on the transport of L-[^{14}C]lactic acid. Moreover, the permeability of [^{3}H]mevalonic acid was not affected by the bicarbonate ion gradient or the addition of DIDS to the transport buffer. Further, mevalonic acid transport was not saturable as shown by the apparent lack of inhibition by unlabeled mevalonic acid. In contrast, the permeability of [^{14}C]salicylic acid and [^{14}C]-benzoic acid was significantly reduced in the absence of the bicarbonate gradient and was inhibited by DIDS and 10 mM of each of these unlabeled compounds in the transport buffer. The transport of [^{3}H]mannitol did not change in the presence of DNP, sodium azide, or DIDS.

Structural Specificity of the Anion-Exchange Transporter—As shown in Table 2, to examine the properties of the anion-exchange transporter responsible for L-lactic acid transfer across Caco-2 cells, the effects of various monocarboxylic acids bearing a hydroxyl group and their derivatives were examined in the presence of a bicarbonate gradient. The permeability of L-[^{14}C]lactic acid was reduced by 2- and 4-hydroxycarboxylic acids (10 mM) such as hydroxyacetic acid, 2-hydroxybutyric acid, and 4-hydroxybutyric acid. It was also reduced by a 5-hydroxycarboxylic acid (10 mM), 5-hydroxydecanoic acid. In contrast, 3-hydroxycarboxylic acids (10 mM), such as 3-hydroxypropionic acid, 3-hydroxybutyric acid, and 3-hydroxy-3-phenylpropionic acid, did not show any significant inhibitory effect on

Table 2—Inhibitory Effect of Monocarboxylic Acids on L-[^{14}C]Lactic Acid Transport[a]

inhibitor	relative permeability (% of control)[b]
hydroxyacetic acid	80.3 ± 3.2*
3-hydroxypropionic acid	95.6 ± 2.0
4-hydroxybutyric acid	72.8 ± 3.5*
2-hydroxybutyric acid	59.9 ± 1.6*
3-hydroxybutyric acid	93.8 ± 12.7
3-hydroxy-3-phenylpropionic acid	89.5 ± 3.9
3-methoxypropionic acid	64.7 ± 1.2*
5-hydroxydecanoic acid	58.0 ± 0.6*

[a] Note: Transport of L-[^{14}C]lactic acid (1 μM) was measured at 37 °C for 60 min by incubating Caco-2 cells in HBSS buffer (apical pH 7.4, basolateral pH 7.4 with bicarbonate) in the presence of each inhibitor. The other incubation conditions were the same as described in the legend to Figure 1. [b] Each value represents the mean ± SEM of three experiments using the same cultivation process of Caco-2 cells and is expressed as percentage of the control. *Significantly different from the control value by Student's *t* test ($p < 0.05$).

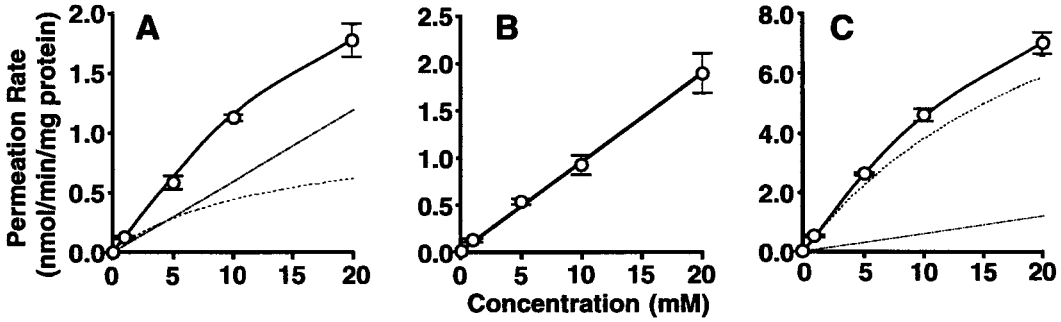

Figure 3—Concentration dependence of the transport of hydroxybutyric acids across Caco-2 cell monolayers. 2-Hydroxybutyric acid (panel A), 3- hydroxybutyric acid (panel B), and 4-hydroxybutyric acid (panel C) were examined. The concentrations of each hydroxybutyric acid (2.5 μM, and 1, 5, 10, and 20 mM) were adjusted by addition of the nonlabeled compound to [^{14}C]hydroxybutyric acid (2.5 μM). The incubation conditions were identical to those described in the legend to Figure 1. The broken lines represent the contribution of the saturable (••••) and nonsaturable (–•–) components to the permeation, calculated from the kinetic parameters obtained as mentioned in the results. Each point represents the mean ± SEM of three experiments using the same culture of Caco-2 cells.

Table 3—Inhibitory Effect of DIDS on [^{14}C]Hydroxybutyric Acids Transport[a]

substrate	permeability coefficient (μL/min/mg protein)[b]	
	control	+2 mM DIDS
[^{14}C]2-hydroxybutyric acid	0.125 ± 0.009	0.084 ± 0.005*
[^{14}C]3-hydroxybutyric acid	0.111 ± 0.008	0.108 ± 0.014
[^{14}C]4-hydroxybutyric acid	0.555 ± 0.032	0.459 ± 0.011*

[a] Note: Transport of [^{14}C]hydroxybutyric acid (2.5 μM) was measured at 37 °C for 60 min by incubating Caco-2 cells in HBSS buffer (apical pH 7.4, basolateral pH 7.4 with bicarbonate) in the presence of each inhibitor. The other incubation conditions were the same as described in the legend to Figure 1. [b] Each value represents the mean ± SEM of three experiments using the same cultivation process of Caco-2 cells. *Significantly different from the control value by Student's *t* test ($p < 0.05$).

the transport of L-[^{14}C]lactic acid, though 10 mM 3-methoxypropionic acid was inhibitory.

Transport Properties of 2-, 3-, and 4-Hydroxybutyric Acids—Figure 3 illustrates the relationship between the initial transport rate of [^{14}C]2-, 3-, and 4-hydroxybutyric acids and the concentration in the medium from 1 μM to 20 mM at the apical pH of 7.4, while the pH on the basolateral side was kept at 7.4 with a 25 mM bicarbonate ion gradient. The results indicate that the permeation of 2- and 4-hydroxybutyric acids involve saturable and nonsaturable processes. Kinetic analysis of the concentration-dependent permeation gave J_{max}, K_t, and k_d values of 2.21 ± 0.22 nmol/min/mg protein, 3.75 ± 0.40 mM, and 0.09 ± 0.01 μL/min/mg protein for 2-hydroxybutyric acid, and 8.44 ± 2.67 nmol/min/mg protein, 23.9 ± 6.41 mM, and 0.12 ± 0.03 μL/min/mg protein for 4-hydroxybutyric acid, respectively. In contrast, the saturable process was not observed for 3-hydroxybutyric acid within the tested concentration range. The first-order rate constant, k_d, was 0.09 ± 0.01 μL/min/mg protein.

Table 3 shows the effects of DIDS on the transport of [^{14}C]-2-, 3-, and 4-hydroxybutyric acids. Permeability of [^{14}C]-2- and 4-hydroxybutyric acids (2.5 μM) in the presence of 2 mM DIDS amounted to 67.1% and 82.6% of the respective control values. The permeability of [^{14}C]-3-hydroxybutyric acid was not affected by DIDS.

Discussion

In the present study, we set out to establish conditions that would maximize the relative activity of anion-exchange transport and to clarify the structural features of substrates required for intestinal transport by examining the transcellular transport of several hydroxy-monocarboxylic acids and their derivatives across monolayers of Caco-2 cells. In the presence of a bicarbonate ion gradient, but not a proton gradient, the concentration dependence of L-lactic acid transport was explained by the participation of a single saturable component, which might be ascribed to the anion-exchange transport mechanism. Under the same conditions, the transport of L-[^{14}C]lactic acid was inhibited by DIDS, which is a typical inhibitor of the anion-exchange transporter. Similar anion antiport activity with bicarbonate was found in the transport of propionic acid and acetic acid.[18,19,3] Previously, we observed enhanced uptake of nicotinic acid when an outward-directed bicarbonate gradient was imposed in isolated jejunal BBMVs.[11] It appears that similar anion-exchange transport mechanisms are involved in the permeation and absorption of monocarboxylic acids in the human intestine.

Mevalonic acid, a 3-hydroxycarboxylic acid, had no effect on the transport of L-[^{14}C]lactic acid in the presence of a bicarbonate ion gradient. In addition, as our previous study using rabbit intestinal BBMVs[11] indicated, the permeation of [^{3}H]mevalonic acid was not affected by the bicarbonate gradient or DIDS. The transport of L-[^{14}C]lactic acid was also inhibited by 2-, 4-, and 5-hydroxycarboxylic acids but not by 3-hydroxycarboxylic acid. These observations suggested that the position of hydroxylation might be an important determinant of monocarboxylate transport by the anion antiport system. Accordingly, we compared the transport of several hydroxycarboxylic acids. The concentration dependence of the 2- and 4-hydroxybutyric acid transport indicated that the permeation involves saturable and nonsaturable processes, whereas that of 3-hydroxybutyric acid was nonsaturable. Since the partition coefficients (as hydrophobic/hydrophilic parameters) of these compounds are almost the same (log $D_{7.4}$ 2-hydroxybutyric acid: −2.35, 3-hydroxybutyric acid: −2.72, 4-hydroxybutyric acid: −2.40), differences in hydrophobicity do not account for the result. It is suggested that 3-hydroxycarboxylic acids might not be substrates for the anion-exchange transporter in intestinal epithelial cells. Interestingly, 3-methoxypropionic acid was shown to have affinity for the anion-exchange transporter, because the transport of L-[^{14}C]lactic acid was inhibited by 3-methoxypropionic acid. This result suggests that a hydrogen bond between the 3-hydroxyl group of carboxylate and the transporter protein might block appropriate interaction with the anion-exchange transporter. It was expected that salicylic acid would not be a substrate for the anion-exchange transporter because it could be regarded as a 3-hydroxycarboxylic acids. However, contrary to expectation, the permeability of [^{14}C]salicylic acid was significantly reduced by the bicarbonate ion-free condition, DIDS, and unlabeled salicylic acid. Within the salicylic acid molecule, there is a relatively stable hydrogen bond conjugated with

the aromatic benzene ring, namely between the 3-hydroxyl group and oxygen of the carboxylic group. This is demonstrated by the ^{1}H NMR signals of the 3-hydroxyl group in salicylic acid and in 3-hydroxybutyric acid (salicylic acid: 11.3 ppm, 3-hydroxybutyric acid: 7.02 ppm). This internal hydrogen bond in salicylic acid might be too strong to allow the blocking interaction with transporter proteins, whereas the relatively "flexible" 3-hydroxyl group in other carboxylic acids can interfere with the proper binding of the substrate to the anion-exchange transporters.

Recently, we demonstrated that the anion exchanger AE2, which is present in several tissues,[20−25] transported organic monocarboxylic acids, when the AE2 gene was transiently overexpressed in HEK 293 cells.[12] So, AE2 might be partially responsible for monocarboxylic acid/anion exchange in the intestine, although more studies are needed on this point.

In conclusion, we have established some of the required structural characteristics for substrates of the anion-exchange transporter using Caco-2 cell monolayers. The observations suggest that an anion antiport system is involved in the intestinal absorption of monocarboxylic acids in parallel with a proton gradient-dependent monocarboxylic acid transporter. Therefore, intestinal absorption of many acidic drugs may proceed, at least in part, via the anion-exchange transporter, rather than by passive diffusion according to the pH-partition hypothesis.

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Acknowledgments

The authors are indebted to Prof. Oyo Mitsunobu, College of Sciences and Engineering, Aoyama Gakuin University, and to Dr. Kazuo Kato and Mr. Kazuyuki Matsuura, Research Center, Mochida Pharmaceutical Co., Ltd., for discussions and valuable advice during preparation of the manuscript.

JS9900093