Proton Gradient-Dependent Transport of Valproic Acid in Human Placental Brush-Border Membrane Vesicles

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Purpose. To investigate the transport mechanism of valproic acid across the human placenta, we used human placental brush-border membrane vesicles and compared them with that of lactic acid.
Methods. Transport of [^{3}H]valproic acid and [^{14}C]lactic acid was measured by using human placental brush-border membrane vesicles.
Results. The uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles were greatly stimulated at acidic extravesicular pH. The uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid were inhibited by various fatty acids, *p*-chloromercuribenzene sulfonate, α-cyano-4-hydroxycinnamate, and FCCP. A kinetic analysis showed that it was saturable, with Michaelis constants (Kt) of 1.04 ± 0.41 mM and 1.71 ± 0.33 mM for [^{3}H]valproic acid and [^{14}C]lactic acid, respectively. Furthermore, lactic acid competitively inhibited [^{3}H]valproic acid uptake and *vice versa.*
Conclusion. These results suggest that the transport of valproic acid across the microvillous membrane of human placenta is mediated by a proton-linked transport system that also transports lactic acid. However, some inhibitors differentially inhibited the uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid, suggesting that other transport systems may also contribute to the elevated fetal blood concentration of valproic acid in gravida.

KEY WORDS: human placenta; transport mechanism; valproic acid; lactic acid.

INTRODUCTION

The placenta plays an important role in exchanging various compounds between mother and fetus (1). Because the fetal blood contacts the maternal circulation only at the placenta, the fetus receives essential nutrients from the mother and transfers spodogenous compounds to the mother across the placenta. The trophoblast cells, which cover placental microvilli with tight junctions and are in contact with the maternal blood, are considered to act as a mother-fetus barrier.

The transport mechanisms of nutrients, such as amino acids, vitamins, and saccharides, across the placenta have been well characterized (2). For amino acids, several transport systems have been reported to function at the placenta (3). For saccharides, facilitated transport systems, GLUT 1 and GLUT 3, were found in the placenta (4). Moreover, several studies revealed the presence of transport systems for thiamine (5), biotin, and pantothenic acid (6) at the brush-border membrane of trophoblast cells. Fetus-derived spodogenous bile acids are transported from fetus to mother in an energy-dependent manner (7). Furthermore, the expression and function of P-glycoprotein, an ATP-dependent efflux transporter, have been identified in the placenta (8).

However, only limited studies have been conducted to elucidate the transport mechanisms of drugs. Maternally administered drugs reach to the fetus across the placenta, but the concentration of drugs in the fetal blood is not always equivalent to the maternal concentration, *ie.,* some drugs are extensively distributed into the fetus, and some are not (9,10). Therefore, to reduce the risk of drug toxicity to the fetus, it is essential to investigate the transport mechanism of drugs across the placenta.

Antiepileptics have been used continuously even during pregnancy and are known to cross the placenta and reach the fetus (11). Valproic acid is one of the most widely used antiepileptics and is used in gravida. However, several studies have shown that valproic acid may induce severe fetal malformation, including spondyloschisis (12,13). Although valproic acid is an anionic drug with pKa of 4.9 and is almost completely ionized under physiologic conditions, it is extensively distributed to the fetus with the concentration ratio in the fetal and maternal blood being 1.7 (11).

Lactic acid is an essential nutrient for the fetus; it is taken into the fetal brain and cardiac myocytes as an energy source or contributes to the maturation of the fetal lung *via* fatty acid synthesis. A proton-linked specific transport system was reported to contribute to the transport of lactic acid across the placenta in rats and humans (14,15). Several monocarboxylic acid transporters (MCT) have recently been cloned and mRNAs of MCT 1, 3, 4, 5, 6, and 7 were found in the placenta (16). It was reported that introduction of MCT1 or MCT2 into *Xenopus laevis* oocytes greatly enhances the uptake of lactic acid, suggesting that lactic acid is one of the substrates of the MCT family (17). Moreover, the uptake of lactic acid into oocytes expressing MCT1 was inhibited by valproic acid (18). These findings suggest that valproic acid might be actively transported into the fetus *via* the placenta by lactic acid transporters of the MCT family, resulting in an elevated concentration of valproic acid in the fetal blood.

The present study was designed to investigate the transport mechanism of valproic acid across the placenta in comparison with that of lactic acid, using human placental brush-border membrane vesicles as an experimental tool so that the driving force of transport could be easily adjusted, and drug metabolism could be excluded.

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ABBREVIATIONS: AP, alkaline phosphatase; 4-CHC, α-cyano-4-hydroxycinnamate; *p*CMBS, *p*-chloromercuribenzene sulfonate; DMSO, dimethylsulfoxide; γ-GTP, γ-glutamyl transpeptidase; MCT, monocarboxylic acid transporters.

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MATERIALS AND METHODS

Materials

[4,5-^{3}H]Valproic acid (55 Ci/mmol) and [U-^{14}C]lactic acid (116 mCi/mmol) were purchased from Moravek Biochemicals Inc. (Brea, California) and [2,3-^{3}H]L-alanine (52 Ci/mmol) was purchased from Amersham International, plc (Buckinghamshire, UK). Lactic acid, acetic acid, propionic acid, butyric acid, and pyruvic acid were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Valproic acid, hexanoic acid, octanoic acid, α-cyano-4-hydroxycinnamate (α-CHC), *p*-chloromercuribenzene sulfonate (*p*CMBS), DIDS, phloretin, and FCCP were purchased from SIGMA Chemical Co. (St. Louis, MO). All other chemicals were commercial products of reagent grade.

Preparation of Human Placental Brush-Border Membrane Vesicles

The human placental brush-border membrane vesicles were prepared by the method described by Smith *et al* (19) with minor modifications. Briefly, human term placentas from uncomplicated pregnancies were obtained within 15 min after vaginal or cesarean delivery and placed in 0.9% NaCl at 4°C. After removal of the cord, amniochorion, and decidua, placental tissue was cut from the maternal side and washed in 300 mM mannitol, 10 mM HEPES-Tris at pH 7.4 (MHT buffer). The mince was stirred for 1 h to loosen the microvilli and filtered through two layers of woven cotton gauze. An aliquot of this starting mince was taken for enzyme analysis. The filtrate was centrifuged at 800 × g for 10 min. The pellet was discarded, and $MgCl_2$ was added to the supernatant to give a final concentration of 10 mM. After 10 min, with occasional stirring, the supernatant was centrifuged at 10,500 × g for 10 min. The pellet was discarded, and the supernatant was centrifuged at 20,000 × g for 20 min. The pellet from this run was suspended in MHT buffer with a 25-gauge syringe needle. All the subsequent procedures were performed at 4°C.

Enzymatic Analysis and Functional Validation

Purity was evaluated by comparing the specific enzyme activity in human placental brush-border membrane vesicles with that in the homogenate. Alkaline phosphatase (AP) and γ-glutamyl transpeptidase (γ-GTP) activities were selected as markers of brush-border membrane (20,21). Protein was quantified by the method of Lowry *et al* (22). The function of brush-border membrane vesicles was validated by measuring uptake of L-alanine, as reported (10,23).

Uptake Studies

The uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles were measured by using a rapid filtration technique (24). Generally, the uptake was started by adding the extravesicular buffer to 50–60 μg of protein of membrane vesicle suspension. Extravesicular buffer consisted of each radiolabeled substrate: both 10 mM HEPES-Tris (pH 7.0–7.4) or MES-Tris (pH 5.5–6.5), and D-mannitol to adjust the osmotic pressure. Transport conditions are described in the figure legends. We used specific inhibitors for lactic acid transport and for the proton-linked transporter to investigate their effects on the uptake of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles. *p*CMBS, 4-CHC, phloretin, DIDS, and FCCP were used as selective inhibitors, an organomercurial thiol reagent, a potent aromatic monocarboxylate inhibitor, a potent inhibitor of the erythrocyte monocarboxylate transporter, and an anionic anion-exchange inhibitor and proton ionophore, respectively (25–31). At an appropriate time after addition of 1 mL of ice-cold stop buffer, the sample was filtered under vacuum through a Millipore filter (HAWP 0.45 μm; Millipore Intertech, MA) and washed twice with 4 mL of ice-cold stop buffer. The stop buffer consisted of extravesicular buffer plus 10 mM D-mannitol without radiolabeled substrate. Nonspecific binding was determined similarly except that 1 mL of ice-cold stop buffer was added to the membrane suspension before adding extravesicular buffer. The radioactivity remaining on the filter was counted in a liquid scintillation counter (LC 6500; Beckman Instruments, Inc., CA), and the uptake of substrates was calculated.

Data Analysis

To estimate the specific uptake of substrate, correction was made for nonspecific binding. To determine the kinetic parameters of [^{3}H]valproic acid and [^{14}C]lactic acid uptake into brush-border membrane vesicles, curve fitting to Eq. (1) was done by least-squares nonlinear regression analysis (MULTI) (32).

$$J = Jmax \times S/(Kt + S) + kd \times S \quad (1)$$

where J and S represent the transport rate and concentration of substrate, respectively. Jmax, Kt, and Kd represent the maximum transport rate, affinity for the transporter and rate constant for nonsaturable transport. For data obtained in the presence of inhibitor, curve fitting to the Eq. (2) was done in the same way.

$$J = Jmax \times S/\{Kt \times (1 + I/Ki) + S\} + kd \times S \quad (2)$$

where Ki and I represent the inhibition constant and the concentration of inhibitor, respectively.

Student's *t* test or ANOVA followed by Duncan's test was used to determine the statistical significance of differences.

RESULTS

Purity of Brush-Border Membrane Vesicles

The AP activities of brush-border membrane vesicles and homogenate were 12.6 ± 1.95 and 0.625 ± 0.111 μmol/min/mg protein, and the γ-GTP activities were 103 ± 8.96 and 6.55 ± 1.28 IU/g protein, respectively. The analyses revealed 22.6-fold and 22.9-fold enrichment of AP and γ-GTP in brush-border membrane vesicles over homogenate, respectively. The amounts of protein in brush-border membrane vesicles and homogenate were 0.103 ± 0.006 and 23.0 ± 5.83 mg protein/g placenta wet weight, respectively, showing that 0.45% of the homogenate protein was recovered as brush-border membrane vesicles.

Functional Validation

The time courses of the uptake of 100 μM [^{3}H]L-alanine into brush-border membrane vesicles in the presence of inward Na^+ and K^+ gradients are presented in Fig. 1. The initial uptake of [^{3}H]L-alanine was significantly stimulated, and transient uphill transport was found in the presence of an inward Na^+ gradient compared with the K^+ gradient. The maximum uptake clearance of [^{3}H]L-alanine at 1 min in the presence of a Na^+ gradient was 4.1-fold higher than that in its absence. As shown in the inset of Fig. 1, the uptake of [^{3}H]L-alanine declined with increase in the extravesicular osmotic pressure. Extrapolation of L-alanine uptake indicated a small amount of membrane binding.

Time Courses and pH-Dependent Uptakes of [^{3}H]Valproic Acid and [^{3}H]Lactic Acid into Brush-Border Membrane Vesicles

Fig. 2 shows the uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles at the extravesicular pH 7.4, 6.5, and 5.5. When the intravesicular pH was fixed at 7.4, the uptake of [^{3}H]valproic acid was increased at acidic extravesicular pH, and transient uphill transport was shown at extravesicular pH 5.5. The maximum uptake clearances at extravesicular pH 5.5 and 6.5 were 16 and 2.2 times greater than without the pH gradient (pH 7.4), when assessed at 10 sec. Similarly, for [^{14}C]lactic acid, 16- and 3.9-fold greater uptake clearances were observed at extravesicular pH 5.5 and 6.5 compared with that in the absence of a pH gradient at 30 sec.

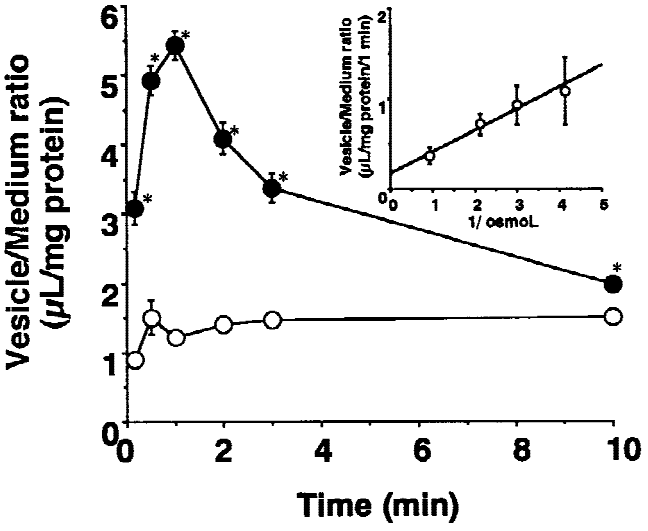

Fig. 1. Effects of an inwardly directed Na^+ gradient on the uptake of [^{3}H]L-alanine into brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]L-alanine (100 μM) was investigated at 37°C in either 100 mM KCl (○) or NaCl (●) containing 10 mM HEPES-Tris buffer (pH 7.4). Each solution contained an appropriate concentration of mannitol to be isotonic. Each point represents the mean ± SEM of four experiments. Significant differences from the control were determined by using Student's *t* test (*$P < 0.05$). Inset: Effects of osmolarity on the uptake of [^{3}H]L-alanine into brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]L-alanine (100 μM) was examined at 37°C in 100 mM NaCl containing 10 mM HEPES-Tris (pH 7.4) and increasing concentrations of mannitol.

Fig. 3 presents the pH profiles of the uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles. The uptake clearances of both compounds were stimulated under an acidic extravesicular condition. This pH-dependency was attenuated in the presence of excess substrate outside. The uptake clearances of [^{3}H]valproic acid and [^{14}C]lactic acid at pH 5.5 were 6.9 times higher than those in the presence of an excess amount of unlabeled substrate (25 mM valproic acid or 50 mM lactic acid).

Effects of Fatty Acids on the Uptake of [^{3}H]Valproic Acid and [^{3}H]Lactic Acid into Brush-Border Membrane Vesicles

Table I presents the effects of short- and medium-chain fatty acids on the uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles. The initial uptake of [^{3}H]valproic acid was inhibited by all the fatty acids examined in a concentration-dependent manner in the presence of a pH gradient. Medium-chain fatty acids, such as hexanoic acid and octanoic acid, potently inhibited the uptake of [^{3}H]valproic acid. The initial uptake of [^{14}C]lactic acid was also inhibited by all the fatty acids examined in a concentration-dependent manner in the presence of a pH gradient and was most potently inhibited by 50 mM acetic acid.

Effects of Various Candidate Inhibitors on the Uptakes of [^{3}H]Valproic Acid and [^{3}H]Lactic Acid into Brush-Border Membrane Vesicles

Initial uptake of [^{3}H]valproic acid was inhibited by *p*CMBS (2, 10 mM), 4-CHC (2, 12.5 mM), DIDS (0.5, 2.5 mM), and FCCP (0.01, 0.025 mM) in a concentration-dependent manner but not affected by phloretin (0.1, 0.5 mM) (Table II). The initial uptake of [^{14}C]lactic acid was inhibited by all the inhibitors examined, except DIDS, in a concentration-dependent manner (Table II).

Kinetics

Fig. 4 shows the concentration-dependent uptakes of [^{3}H]valproic acid and [^{14}C]lactic acid into brush-border membrane vesicles and the effects of unlabeled lactic acid and valproic acid, respectively. The initial uptake of [^{3}H]valproic acid was concentration dependent and saturable, being described by Eq. (1). The kinetic parameters obtained were Kt of 1.04 ± 0.41 mM, Jmax of 4.72 ± 1.27 nmol/mg protein/30 sec and Kd of 0.77 ± 0.05 μL/mg protein/30 sec (Fig. 4). In the presence of 7.5 mM lactic acid, the kinetics followed Eq. (2). Simultaneous fitting of the data to Eq. (1) and (2) provided the Ki value of 1.51 ± 0.51 mM. For [^{14}C]lactic acid, the kinetic parameters obtained were Kt of 1.71 ± 0.330 mM, Jmax of 8.59 ± 1.43 nmol/mg protein/5 sec, and Kd of 0.76 ± 0.070 μL/mg protein/5 sec (Table III). In the presence of excess valproic acid (7.5 mM), Ki was estimated to be 1.65 ± 0.270 mM. The inset of Fig. 4 shows the Eadie-Hofstee plot of the saturable component, which was calculated by subtracting the nonsaturable component (Kd × S) from the total uptake.

DISCUSSION

Valproic acid is one of the most widely used antiepileptics, but the fetal toxicity is a serious problem (12,13). As the

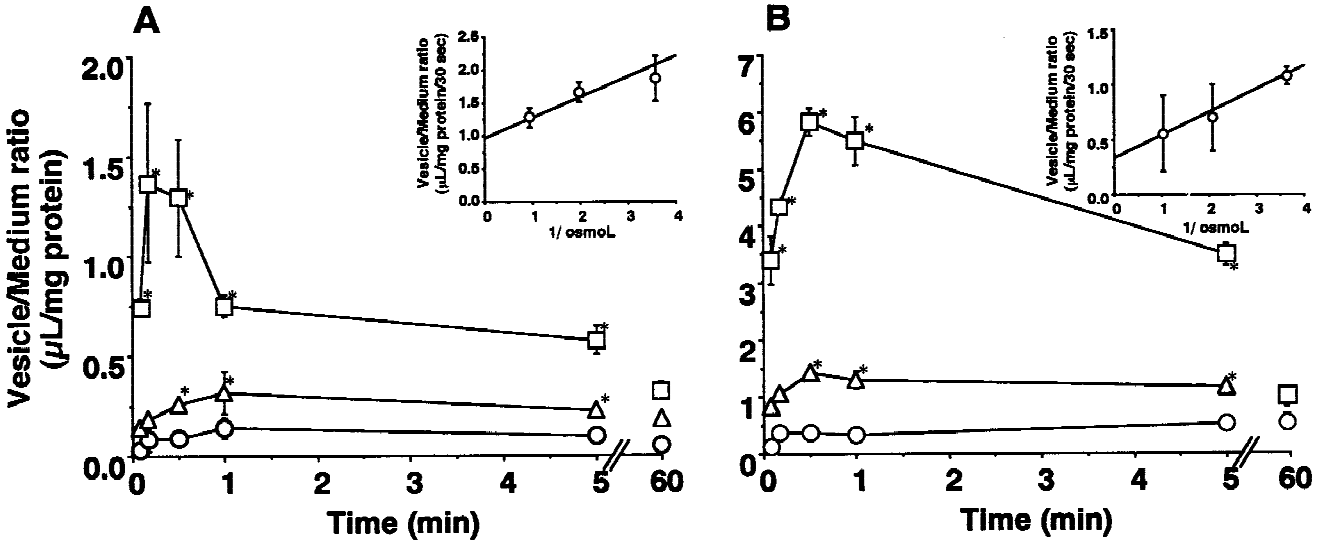

Fig. 2. Effects of extravesicular pH on [^{3}H]valproic acid (A) and [^{14}C]lactic acid (B) uptake into brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]valproic acid (10 μM) or [^{14}C]lactic acid (1 mM) was investigated at 37°C in either 10 mM HEPES-Tris buffer (pH 7.4, ○) or 10 mM MES-Tris buffer (pH 6.5, △; pH 5.5, □). Each solution contained an appropriate concentration of mannitol to be isotonic. Each point represents the mean ± SEM of four experiments. Significant differences from the control were determined by using Student's *t* test (*$P < 0.05$). Inset: Effects of osmolarity on the uptakes of [^{3}H]valproic acid (A) and [^{14}C]lactic acid (B) into brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]valproic acid (10 μM) or [^{14}C]lactic acid (1 mM) was examined for 30 s at 37°C in 10 mM MES-Tris (pH 5.5) containing increasing concentrations of mannitol.

fetal blood concentration of valproic acid becomes higher than the maternal blood concentration (12,13), the extent of fetal toxicity may be explained in extensive distribution of valproic acid into fetal blood. Therefore, we evaluated the transport mechanisms of valproic acid across the placenta, in comparison with those of lactic acid, for which the transport systems in the placenta have been well investigated.

We first examined the activities of AP and γ-GTP to confirm the purity of the brush-border membrane vesicle preparation. The enrichment of each enzymatic activities was enhanced 20-fold over the homogenate, being comparable to previous results (10,23). Thus, our brush-border membrane preparation was considered to be highly purified. Furthermore, in agreement with previous reports (10,23), Na^+-

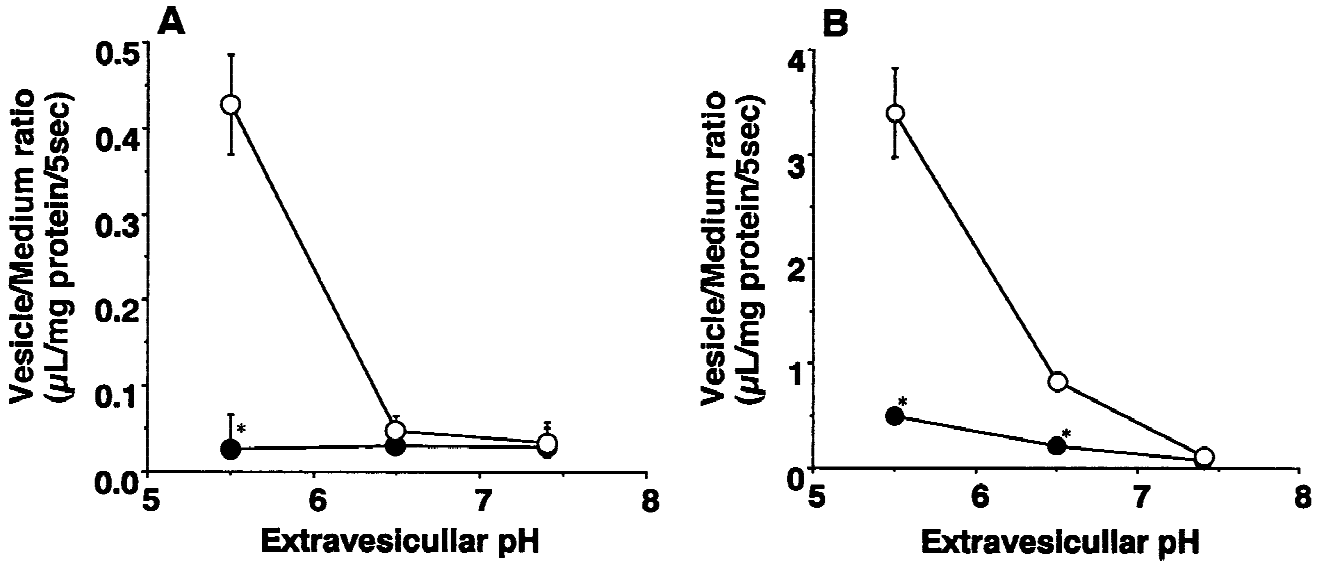

Fig. 3. Extravesicular pH-dependent uptake of [^{3}H]valproic acid (A) and [^{14}C]lactic acid (B) into brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptakes of [^{3}H]valproic acid (10 μM, ○; 25 mM, ●) and [^{14}C]lactic acid (1 mM, ○; 50 mM, ●) were examined at 37°C for 5 sec in either 10 mM HEPES-Tris buffer (pH 7.4) or 10 mM MES-Tris buffer (pH 6.5 and pH 5.5). Each solution contained an appropriate concentration of mannitol to be isotonic. Each point represents the mean ± SEM of four experiments. Significant differences from the control were determined by using Student's *t* test (*$P < 0.05$).

Table I. Effects of Fatty Acids on the Uptakes of [^{3}H]Valproic Acid and [^{14}C]Lactic Acid into Brush-Border Membrane Vesicles

	Relative uptake (% of control)	
Inhibitors (mM)	[^{3}H]valproic acid	[^{14}C]lactic acid
Acetic acid		
5	68.8 ± 13.1	74.8 ± 3.42*
50	35.7 ± 15.4*	(not detected)
Lactic acid		
5	63.8 ± 9.44	72.3 ± 1.71*
50	34.4 ± 6.07*	29.3 ± 1.97*
Propionic acid		
5	72.3 ± 20.5	90.9 ± 4.84
50	32.8 ± 17.5*	30.1 ± 0.44*
Butyric acid		
5	52.7 ± 26.9*	88.5 ± 6.07
50	23.0 ± 19.4*	35.2 ± 1.60*
Pyruvic acid		
5	87.5 ± 15.9	87.0 ± 4.40
50	46.4 ± 14.3	29.9 ± 2.20*
Hexanoic acid		
5	15.5 ± 1.96*	69.2 ± 2.67
50	(not detected)	31.1 ± 2.55*
Octanoic acid		
5	30.7 ± 20.3*	69.6 ± 2.67
50	(not detected)	38.6 ± 1.76*

Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]valproic acid (10 μM) and [^{14}C]lactic acid (1 mM) were examined at 37°C for 5 sec in 10 mM MES-Tris buffer (pH 5.5) and 5 mM or 50 mM inhibitor. Each solution contained an appropriate concentration of mannitol to be isotonic. Each value represents the mean ± SEM of four experiments. Significant differences from the control were determined by using ANOVA followed by Duncan's test (*$P < 0.05$).

dependent L-alanine uptake was shown, indicating that the brush-border membrane vesicles were functional.

The uptakes of valproic acid and lactic acid into brush-border membrane vesicles were examined by a rapid filtration technique (24). To correct for nonspecific binding, the uptake at 4°C at 0 sec was subtracted from the total uptake in general. The effect of the extravesicular osmolarity of buffer on the uptake was examined for each substrate, and the uptake decreased at higher osmolarity (Fig. 2 inset). Therefore, the uptake clearance observed in this study did not reflect binding to the membrane but uptake into intravesicular space.

Lactic acid is transported by proton-linked (pH-dependent) active transport systems in the placenta (15). In our study, the uptake of lactic acid also increased with decrease in the extravesicular pH (Fig. 2B). The uptake of valproic acid was also pH dependent, suggesting that it also requires, a proton gradient. Furthermore, an overshoot phenomenon, which is generally agreed to be a distinct feature of active transport, was observed at acidic extravesicular pH (Fig. 2). The uptake clearance increased at acidic extravesicular pH and was significantly attenuated by high external concentrations of substrates (Fig. 3). Therefore, we concluded that valproic acid, like lactic acid, is transported by pH-dependent active transport system(s) in brush-border membrane. In the steady-state condition, intravesicular pH may be decreased to pH 5.5 at the extravesicular pH 5.5, and the uptakes of valproic acid and lactic acid are determined by the pH partition theory. Therefore, we evaluated the initial uptake rate at the time when proton-gradient exists. On the other hand, Na^+/H^+ exchanger has been found on the brush-border membranes of trophoblast cells (33). The Na^+/H^+ exchanger may provide plausibly the driving force of this active transport. Furthermore, the contribution of passive diffusion was estimated by measuring the initial uptake rate in the presence of excessive concentration of substrate. Therefore, the proton-gradient uptake of substrates was properly evaluated at extravesicular pH 5.5 and intravesicular pH 7.4.

In our present study, the substrate concentrations of lactic acid and valproic acid were adjusted to their physiologic and clinical levels, *i.e.,* 1 mM for lactic acid and 10 μM valproic acid. The therapeutic maternal concentration of unbound valproic acid and the physiologic maternal concentration of lactic acid are considered to be 0.05 mM and 1 mM, respectively, suggesting that the transport of lactic acid from mother to fetus may not be inhibited by valproic acid under the clinical condition. However, the transport of valproic acid may be affected by lactic acid under physiologic conditions. Because the concentration of lactic acid is affected by exercise or diet, the transport of valproic acid from mother to fetus may possibly be altered by these factors.

Next, we examined the effects of several candidate inhibitors on the pH-dependent uptake of valproic acid and lactic acid into brush-border membrane vesicles. The uptake of lactic acid was inhibited by FCCP (Table II). Nabuchi *et al* (34) reported that the uptake of lactic acid into brush-border membrane vesicles decreased in the presence of FCCP, and the overshoot phenomenon disappeared. The uptake of lactic acid was not affected by DIDS (Table II). Although Balk-

Table II. Effects of Various Candidate Inhibitors on the Uptakes of [^{3}H]Valproic Acid and [^{14}C]Lactic Acid into Brush-Border Membrane Vesicles

	Relative uptake (% of control)	
Inhibitors (mM)	[^{3}H]valproic acid	[^{14}C]lactic acid
*p*CMBS		
2	31.6 ± 13.1*	74.6 ± 4.39*
10	17.0 ± 9.86*	50.1 ± 0.20*
4-CHC		
2	75.8 ± 10.4*	84.8 ± 4.97
12.5	46.0 ± 11.5*	59.7 ± 1.04*
Phloretin		
0.1	102 ± 16.1	76.7 ± 3.54*
0.5	102 ± 14.5	85.5 ± 1.36
DIDS		
0.5	51.1 ± 8.79*	94.4 ± 3.99
2.5	32.5 ± 2.76*	81.6 ± 0.59
FCCP		
0.01	79.8 ± 10.3	78.4 ± 1.71
0.025	31.2 ± 9.40*	69.4 ± 1.80*

Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). The uptake of [^{3}H]valproic acid (10 μM) and [^{14}C]lactic acid (1 mM) were examined at 37°C for 5 sec in 10 mM MES-Tris buffer (pH 5.5) and appropriate concentrations of inhibitors in the presence of 0.5% dimethylsulfoxide (DMSO) (except *p*CMBS). Each solution contained an appropriate concentration of mannitol to be isotonic. Each value represents the mean ± SEM of four experiments. Significant differences from the control were determined by using ANOVA followed by Duncan's test (*$P < 0.05$).

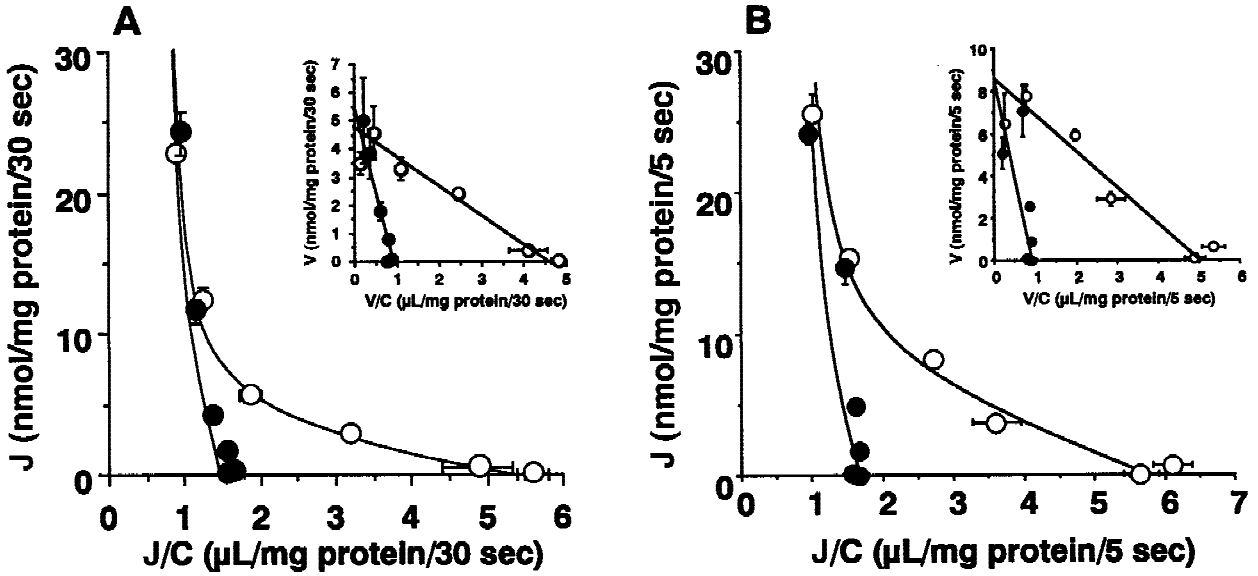

Fig. 4. Eadie-Hofstee plots of the uptake of [^{3}H]valproic acid into brush-border membrane vesicles in the presence or absence of lactic acid. The uptake of [^{3}H]valproic acid was investigated at 37°C in 10 mM MES-Tris buffer (pH 5.5) containing various concentrations (0.01, 0.1, 1, 3, 10, or 25 mM) of unlabeled valproic acid in the absence (○) or presence (●) of 7.5 mM lactic acid. (B) Eadie-Hofstee plots of the uptake of [^{14}C]lactic acid into brush-border membrane vesicles in the presence or absence of valproic acid. The uptake of [^{14}C]lactic acid was performed at 37°C in 10 mM MES-Tris buffer (pH 5.5) containing various concentrations (0.01, 0.1, 1, 3, 10, or 25 mM) of unlabeled lactic acid in the absence (○) or presence (●) of 7.5 mM valproic acid. Brush-border membrane vesicles were preloaded with 10 mM HEPES-Tris buffer (pH 7.4). Each solution contained an appropriate concentration of mannitol to be isotonic. Inset: The saturable uptake was calculated by subtracting the passive diffusion component from total uptake.

ovetz *et al* (15) reported that DIDS did not affect the uptake of lactic acid, their data showed a tendency for DIDS to inhibit the uptake. As in the case of lactic acid, the uptake of valproic acid was inhibited by FCCP in a concentration-dependent manner (Table II). Therefore, a pH-dependent proton-linked transport system is considered to take part in the uptake of valproic acid, as well as lactic acid. Although the uptake of valproic acid was significantly inhibited in the presence of DIDS, the uptake of lactic acid was not significantly affected (Table II). These results suggest that the DIDS sensitive transport system may contribute to the uptake of valproic acid, but not to that of lactic acid.

Furthermore, to estimate quantitatively the transport mechanisms of valproic acid and lactic acid, we carried out kinetic analyses. The uptake of lactic acid was saturable in the presence of an inward-directed proton-gradient (Fig. 4B), and the kinetic parameters were calculated to be Kt of 1.71 ± 0.33 mM and Jmax of 8.59 ± 1.43 nmol/mg protein/5 sec (Table III). The affinity of lactic acid for the monocarboxylic acid transporter was reported to be 4 mM (15), which is comparable to our present result. The uptake of valproic acid was also saturable (Fig. 4A) with an affinity for the transporter (Kt) of 1.04 ± 0.41 mM, which is similar to that of lactic acid. The Kt value of lactic acid was virtually equivalent to its Ki value for the valproic acid (Table III), suggesting that both compounds are transported by a common transporter, or several transporters with similar affinities. Although Kt value of valproic acid was slightly lower than its Ki value for lactic acid (Table III), the difference is considered to be within the experimental error of the study. However, valproic acid does not necessarily share a common transport system with lactic acid. The contribution of other distinct transport system(s) with similar affinity may not be thoroughly excluded.

Analysis of the inhibitory properties of several monocarboxylic acids revealed that the uptakes of valproic acid and lactic acid were equally inhibited (by 50%) by 50 mM propanoic acid or pyruvic acid. It was reported that the uptake of lactic acid was inhibited 67 or 71% by 10 mM propanoic acid

Table III. Kinetic Parameters for Valproic Acid and Lactic Acid Uptake into Brush-Border Membrane Vesicles

Substrate	Jmax	Kt	kd	Ki	
				Valproic acid	Lactic acid
Valproic acid	4.72 ± 1.27 (nmol/mg protein/30 sec)	1.04 ± 0.41 (mM)	0.770 ± 0.05 (μL/mg protein/30 sec)	—	1.51 ± 0.51[a] (mM)
Lactic acid	8.59 ± 1.43 (nmol/mg protein/5 sec)	1.71 ± 0.33 (mM)	0.764 ± 0.07 (μL/mg protein/5 sec)	1.66 ± 0.27[b] (mM)	—

Estimated value ± SD of the estimated residual weighted sum of squares.
[a] Ki of lactic acid for the uptake of valproic acid.
[b] Ki of valproic acid for the uptake of lactic acid.

and pyruvic acid, respectively (15), which is compatible with our results. However, not all the monocarboxylic acids equally inhibited the uptakes of valproic acid and lactic acid (Table I), *i.e.,* the uptake of valproic acid was more potently inhibited by medium-chain fatty acid such as hexanoic acid and octanoic acid in comparison with that of lactic acid (Table I). Recently it was reported that the transport of valproic acid into rat brain is inhibited particularly by medium-chain fatty acids (35). Although the similar transport system has not been identified in the placenta up to now, such a system may exist and contribute to the transport valproic acid at least in part.

Specific inhibitors lactic acid transport has been characterized mainly in erythrocytes. *p*CMBS is an irreversible inhibitor and 4-CHC and phloretin are reversible inhibitors (15,26,27,36). In our present study, the uptake of lactic acid was inhibited by 15 and 40% in the presence of 2 and 12.5 mM 4-CHC, respectively (Table II). These results are in accordance with the report that the uptake of lactic acid into rat brush-border membrane vesicles from placenta was inhibited by 4 and 42% in the presence of 5 and 25 mM 4-CHC, respectively (14). *p*CMBS (2 mM) and phloretin (0.5 mM) inhibited the uptake of lactic acid by 25 and 15%, respectively (Table II), whereas Ritzhaupt *et al* (37) reported that the uptake of lactic acid into porcine colonic brush-border membrane vesicles was potently inhibited by 63 and 55% by 0.5 mM *p*CMBS and phloretin, respectively. The difference may be due to the difference in organs. *p*CMBS and 4-CHC also inhibited the uptake of valproic acid, as well as lactic acid, in a concentration-dependent manner (Table II), whereas phloretin did not affect the uptake of valproic acid in the placenta, in contrast to other organs. The transport system of valproic acid in the placenta may consist of transporter(s) nonsensitive to phloretin. In other words, a distinct transporter or several transporters with similar affinity, which are all insensitive to phloretin, may transport valproic acid in the placenta.

Recently, several members of the MCT family, which selectively transport monocarboxylic compounds such as lactic acid, were cloned. The expression in the placenta of mRNAs of the MCT1, 3, 4, 5, 6, and 7, except MCT2, was reported (16). Broer *et al* (38) investigated the function of MCT family members by using *Xenopus laevis* oocytes and concluded that the uptake of lactic acid into oocytes expressing MCT was inhibited by *p*CMBS and 4-CHC. The above report is consistent with the results of this study. Therefore, it is likely that MCT molecules are involved in the proton-linked transport of lactic acid and valproic acid in the placenta. Although the inhibitory effects on the uptake of valproic acid and lactic acid were different in the case of several inhibitors such as phloretin, DIDS and medium-chain fatty acids, these differences may be attributed to the contribution of other transport system(s) and/or another isoform(s) of MCT family. Further study is required to clarify the quantitative contribution of each isoform of MCTs and other transporter(s) with similar affinity on the transport of lactic acid and valproic acid.

In conclusion, a proton-linked saturable transport system for valproic acid including the passive diffusion component was identified on the brush-border membrane in human placenta. The characteristics of the saturable system were quite similar to those of MCT family members, whereas several distinct transporters with different sensitivity to medium-chain fatty acids, DIDS, and phloretin may contribute to the transport of valproic acid in human placenta. The operation of these transport systems increases the fetal blood concentration of valproic acid, which may contribute to the teratogenic action of this drug.

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