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Uptake mechanism of valproic acid in human placental choriocarcinoma cell line (BeWo)

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Abstract

Valproic acid is an anticonvulsant widely used for the treatment of epilepsy. However, valproic acid is known to show fetal toxicity, including teratogenicity. In the present study, to elucidate the mechanisms of valproic acid transport across the blood–placental barrier, we carried out transcellular transport and uptake experiments with human placental choriocarcinoma epithelial cells (BeWo cells) in culture. The permeability coefficient of [^{3}H]valproic acid in BeWo cells for the apical-to-basolateral flux was greater than that for the opposite flux, suggesting a higher unidirectional transport in the fetal direction. The uptake of [^{3}H]valproic acid from the apical side was temperature-dependent and enhanced under acidic pH. In the presence of 50 μM carbonyl cyanide *p*-trifluoromethoxylhydrazone, the uptake of [^{3}H]valproic acid was significantly reduced. A metabolic inhibitor, 10 mM sodium azide, also significantly reduced the uptake of [^{3}H]valproic acid. Therefore, valproic acid is actively transported in a pH-dependent manner on the brush-border membrane of BeWo cells. Kinetic analysis of valproic acid uptake revealed the involvement of a non-saturable component and a saturable component. The Michaelis constant for the saturable transport (K_t) was smaller under acidic pH, suggesting a proton-linked active transport mechanism for valproic acid in BeWo cells. In the inhibitory experiments, some short-chain fatty acids, such as acetic acid, lactic acid, propanoic acid and butyric acid, and medium-chain fatty acids, such as hexanoic acid and octanoic acid, inhibited the uptake of [^{3}H]valproic acid. The uptake of [^{3}H]valproic acid was also significantly decreased in the presence of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, salicylic acid and furosemide, which are well-known inhibitors of the anion exchange system. Moreover, *p*-aminohippuric acid significantly reduced the uptake of [^{3}H]valproic acid. These results suggest that an active transport mechanism for valproic acid exists on the brush-border membrane of placental trophoblast cells and operates in a proton-linked manner.

Keywords: BeWo cell; Valproic acid; Epilepsy

1. Introduction

Throughout gestation, the placenta plays an important role in regulating the exchange of various materials between the maternal and fetal circulations (Stulc, 1997). Many investigators have examined the transport mechanisms of nutrients, such as amino acids, vitamins and glucose, across the blood–placental barrier, which consists of trophoblast cells (Hay, 1994). Several transport systems for amino acids, such as Na^+-dependent A or ASC system and the Na^+-independent L system, have been described in trophoblasts (Furesz et al., 1993; Moe et al., 1994; Moe, 1995; Ramamoorthy et al., 1992). Glucose transporter 1 (GLUT 1) is expressed both on the brush-border (maternal side) and the basal (fetal side) membranes, while glucose transporter 3 (GLUT 3) is expressed only on the brush-border membrane (Bissonnette, 1981; Reiid and Boyd, 1994; Hahn and Desoye, 1996). Thiamin (vitamin B1) is transported in exchange with H^+ via a Na^+- and membrane potential-dependent transport system (Grassl, 1998). Biotin, lipoate and pantothenate are co-transported with Na^+ (Hu et al., 1994; Schenker et al., 1992; Prasad et al., 1998). However, only limited studies have been conducted to elucidate the transport of drugs or xenobiotics across the

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blood–placental barrier, even though, when administered during pregnancy, several drugs are known to cross the placenta and reach the developing fetus. The fetal blood concentration of drugs is not always equivalent to the maternal blood concentration (Pacifici and Nottoli, 1995; Van der Aa et al., 1998), suggesting that there are active transport systems for drugs in the placenta. It is especially serious if the fetal blood concentration of a drug becomes greater than the maternal blood concentration (e.g., valproic acid). Therefore, it is important to elucidate the placental transport mechanisms for drugs.

There are many pregnant women with epilepsy. Anticonvulsants administered during pregnancy are known to cross the placenta and reach the developing fetus. In particular, valproic acid is frequently administered for the treatment of all forms of epilepsy during pregnancy, though there is a risk of fetal toxicity including teratogenicity (Malone and D'Alton, 1997; Yerby, 1997). Although valproic acid, with a pK_a value of 4.9, behaves as an anionic ion under physiological pH, it easily crosses the placenta. Its fetal-to-maternal blood concentration ratio is reported to be 1.71 (Ishizaki et al., 1981). Although protein binding and the composition of plasma protein and fatty acids may be important factors (Ishizaki et al., 1981; Johannessen, 1992), the definite factor for the transport of valproic acid from the mother to the fetus has not been fully characterized. The mechanism of valproic acid transport at the blood–brain barrier has been examined in rats and revealed to include one saturable carrier-mediated system and a non-saturable component. The saturable carrier-mediated system for valproic acid may be related to the transport system for short- and medium-chain fatty acids (Adkison et al., 1996; Adkison and Shen, 1995; Naora and Shen, 1995). Recently, in placental trophoblast cells, Utoguchi and Audus (2000) reported that valproic acid is transported by a saturable transport system in a proton-dependent manner. However, they did not examine the pH dependence or the effects of various inhibitors, so that detailed characteristics of valproic acid transport across the placenta remain unknown.

In the present study, we sought to elucidate the mechanism for valproic acid transport across the blood–placental barrier in detail by means of transcellular transport and uptake experiments with human placental choriocarcinoma epithelial cells (BeWo cells) in culture.

2. Methods

2.1. Materials and reagents

[4,5-^{3}H]Valproic acid (55 Ci/mmol) and [1-^{14}C]D-mannitol (53 Ci/mmol) were purchased from Moravek Biochemicals (California, USA). [2,3-^{3}H]L-Alanine (52 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK), and 3-*O*-[methyl-^{3}H]-D-glucose (81.5 Ci/mmol) from NEN Life Science Products, (Boston, MA, USA). 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 2-(*N*-morpholineo) ethanesulfonic acid (MES), sodium azide, propanoic acid, butyric acid, lignoceric acid, lactic acid, pyruvic acid, succinic acid, *p*-aminohippuric acid (PAH) and salicylic acid were purchased from Nacalai Tesque (Kyoto, Japan). Valproic acid, phloretin, carbonyl cyanide *p*-trifluoromethoxylhydrazone (FCCP), hexanoic acid, octanoic acid, stearic acid, glutaric acid, probenecid, furosemide and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used in the experiments were of reagent grade. BeWo cells were purchased from American Type Culture Collection (Rockville, MD, USA).

2.2. Cell culture

BeWo cells were cultured in the F12K modification of Ham's medium (Sigma) supplemented with 10% fetal calf serum, 50 mg/ml streptomycin, 50 units/ml penicillin G, at 37°C under 5% CO2/95% air (Pattilo et al., 1986). For the uptake or transport study, the cells were seeded at 4×10^4 cells/well on 96-well multi dishes (Nunc, Denmark) or 1×10^7 cells/well on a polycarbonate membrane (3.0 μm pore size) Transwell™ cluster (Corning COSTER JAPAN, Japan). The cells were grown for 4 or 12 days and used for the uptake or transport study. In the transport study, we used BeWo cells after monitoring the membrane resistance across the BeWo monolayer and used the transport of mannitol as a paracellular transport marker.

2.3. Transcellular transport of [^{3}H]valproic acid across BeWo cells

After reaching confluence, BeWo cells were washed three times with transport buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose and 25 mM HEPES for pH 7.4; the pH was adjusted with NaOH at 37°C) before the transcellular transport study. Transport buffer was added to both the receiver and donor sides. [^{3}H]Valproic acid (500 μM) and [^{14}C]mannitol (800 nM) were added to the donor and receiver sides, of the cell insert, respectively. The sampling times were 0.167, 0.5, 1, 3, 6, 9, 15, 30, 60, 90 and 120 min after incubation with each drug at 37°C. The transport of [^{3}H]valproic acid and [^{14}C]mannitol was quantified by adding 4 ml of scintillation cocktail (Clear-sol I, Nacalai Tesque) and measured using a liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA, USA). The real permeability coefficient (P_{trans}) of [^{3}H]valproic acid was calculated with the following equation:

$$1/P_{app} = 1/P_{filter} + 1/P_{trans} \quad (1)$$

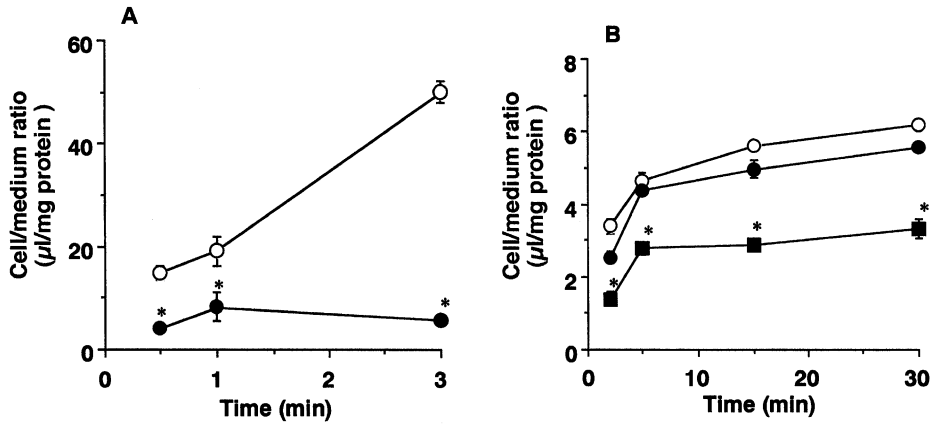

Fig. 1. (A) The initial uptake of 100 μM [^{3}H]L-alanine by BeWo cells at pH 7.4. The uptake of [^{3}H]L-alanine was measured at 37°C in the presence (open circles) and absence of Na^+ (closed circles). Each point represents the mean ± S.E.M. of four experiments. Significant differences from the control were identified by Student's *t*-test (*: $P < 0.05$). (B) The uptake of 500 μM 3-*O*-[methyl-^{3}H]D-glucose by BeWo cells was measured at 37°C in the presence (open circles) and absence of Na^+ (closed circles), and in the presence of phloretin (closed squares). Each point represents the mean ± S.E.M. of four experiments. Significant differences from the control were identified by Student's *t*-test (*: $P < 0.05$).

where P_{app} and P_{filter} are the apparent permeability coefficients estimated in the transport study in the presence and absence of BeWo cells, respectively.

2.4. Intracellular volume of BeWo cells and uptake activity of BeWo cells

The uptake activity of BeWo cells was confirmed by uptake experiments with [^{3}H]L-alanine in uptake buffer containing 100 μM [^{3}H]L-alanine in the presence or absence of Na^+. The intracellular volume of BeWo cells was calculated from the uptake of 3-*O*-[methyl-^{3}H]D-glucose, which was measured in the uptake buffer containing 500 μM 3-*O*-[methyl-^{3}H]D-glucose in the presence of 1 mM phloretin, an inhibitor of the Na^+-independent glucose transporter, with or without Na^+.

2.5. Cellular uptake of [^{3}H]valproic acid by BeWo cells

The culture medium was removed and cells were washed three times with 100 μl of uptake buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM D-glucose and 25 mM HEPES for pH 7.4 or 25 mM MES for pH 6.5; the pH was adjusted with NaOH) at 37°C or 4°C. Uptake experiments were performed in uptake buffer containing [^{3}H]valproic acid (500 μM) in the absence or presence of metabolic inhibitor, unlabeled valproic acid, fatty acids, or inhibitors at 37°C or 4°C. In the metabolic inhibition experiments, cells were preincubated with uptake buffer containing 10 mM sodium azide for 10 min. The cells were washed four times with 100 μl of ice-cold buffer to stop uptake. Cells were dissolved in 200 μl of 3 N NaOH and neutralized with 100 μl of 6 N HCl, and

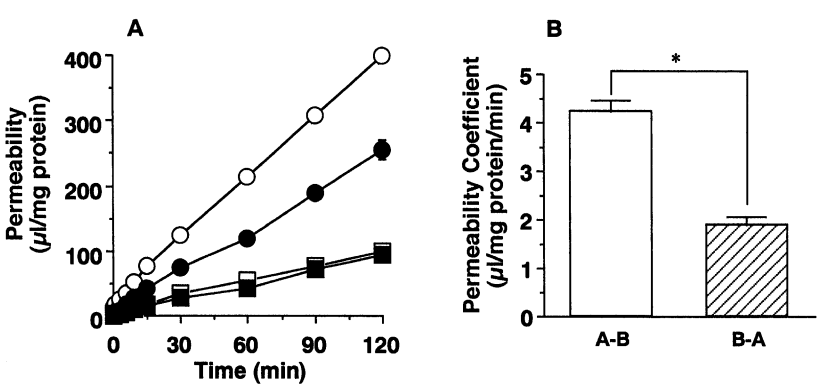

Fig. 2. The transcellular transport of [^{3}H]valproic acid across BeWo cell monolayer. The permeability (A) and the permeability coefficients (B) of 500 μM [^{3}H]valproic acid were measured as described in Methods. After [^{3}H]valproic acid was applied to the apical side (open circles: apical-to-basolateral side) or the basolateral side (closed circles: basolateral-to-apical side), and 800 nM [^{14}C]mannitol to the apical side (open squares: apical-to-basolateral side) or the basolateral side (closed squares: basolateral-to-apical side), an aliquot was withdrawn from the basolateral side or the apical side, respectively. A-to-B and B-to-A show the apical-to-basolateral and basolateral-to-apical fluxes. Each point represents the mean ± S.E.M. of three experiments. Significant differences from control were identified by Student's *t*-test (*: $P < 0.05$).

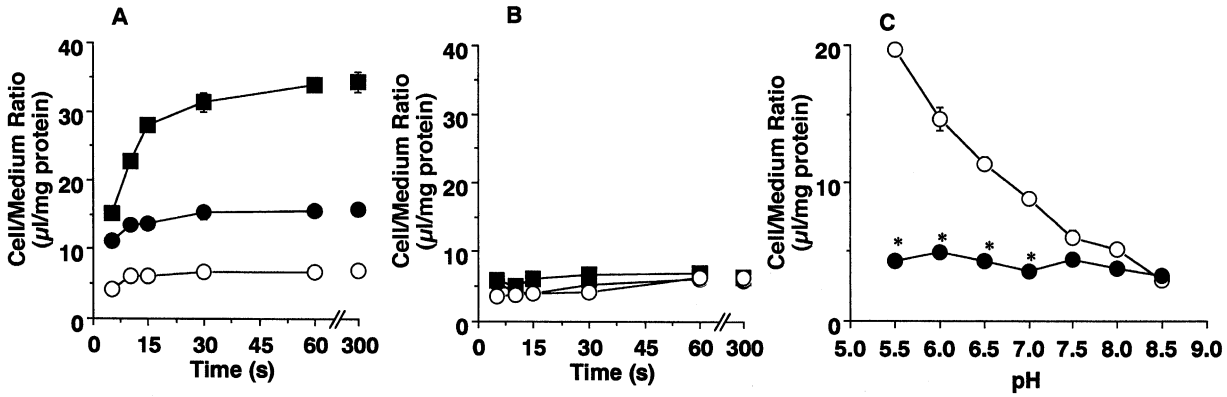

Fig. 3. The time course of 500 μM [^{3}H]valproic acid uptake by BeWo cells at different pH at 37°C (A) or 4°C (B) and the pH-dependent initial uptake of valproic acid at 37°C (C). (A) or (B) The uptake of [^{3}H]valproic acid by BeWo cells at 37°C or 4°C. The uptake of [^{3}H]valproic acid was measured at pH 7.4 (open circles), 6.5 (closed circles) and 5.5 (closed squires), respectively. (C) pH dependence and the effects of self-inhibition on the initial uptake of [^{3}H]valproic acid at 10 s by BeWo cells. The cell/medium ratio at 10 s of [^{3}H]valproic acid was measured by incubating BeWo cells in uptake buffer in the absence (open circles) or presence of 10 mM unlabeled valproic acid (closed circles). Each point represents the mean ± S.E.M. of four experiments. Significant differences from control were identified by Student's *t*-test (*: $P < 0.05$).

scintillation cocktail (Clear-sol I, Nacalai Tesque) was added. The radioactivity of intracellular [^{3}H]valproic acid was then determined with a liquid scintillation counter (LS6500, Beckman Instruments). The amount of protein in the cells was measured by Lowry's method (Lowry et al., 1951). The uptake of [^{3}H]valproic acid is expressed as the cell/medium ratio calculated from the intracellular uptake per mg protein of the cells relative to the initial drug concentration (μl/mg protein).

2.6. Data analysis

To estimate the kinetic parameters of [^{3}H]valproic acid uptake in BeWo cells, the data set for the uptake rate (J) at various concentrations of valproic acid was fitted to the Eq. (2), which represents a kinetic model with saturable and non-saturable components, using the non-linear least-squares regression analysis program, MULTI (Yamaoka et al., 1981):

$$J = J_{max} C/(K_t + C) + k_d C \quad (2)$$

where J_{max} is the maximum uptake rate for the saturable component, K_t is the Michaelis constant, k_d is the first-order constant for the non-saturable component and C is the concentration of valproic acid. All of the data are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) followed by Duncan's test. The difference be-

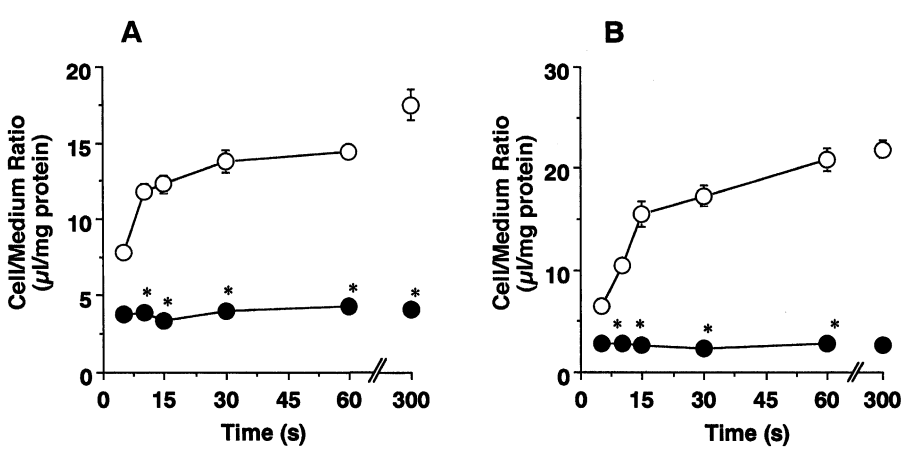

Fig. 4. Effect of metabolic inhibitor (10 mM sodium azide) (A) and 50 μM carbonyl cyanide *p*-trifluoromethoxylhydrazone (FCCP) (B) on the uptake of 500 μM [^{3}H]valproic acid by BeWo cells at pH 6.5. Each point represents the mean ± S.E.M. of four experiments. Significant differences from control were identified by Student's *t*-test (*: $P < 0.05$).

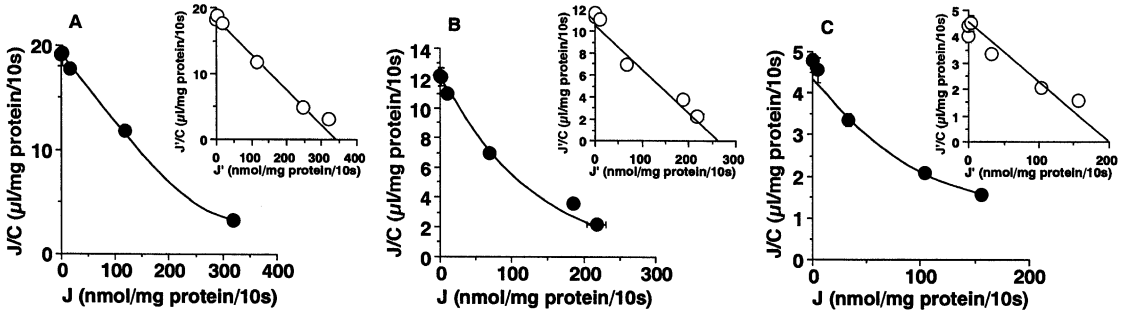

Fig. 5. Concentration dependence of the initial uptake of valproic acid by BeWo cells. Eadie–Hofstee plots of the uptake of valproic acid at pH 5.5 (A), 6.5 (B) and 7.4 (C) are presented. Each point represents the mean ± S.E.M. of four experiments. Curves for total uptake were calculated using the parameters obtained from nonlinear regression analysis. Each inset shows the uptake by the saturation component, which was calculated by subtracting the passive diffusion component from the total uptake. Each point represents the mean ± S.E.M. of four experiments.

tween means was considered to be significant when the *P* value was less than 0.05.

3. Results

3.1. The uptake of [^{3}H]L-alanine and 3-O-[methyl-^{3}H]D-glucose by BeWo cells

The initial uptake of [^{3}H]L-alanine by BeWo cells was reduced in the absence of Na^+ (Fig. 1A). In contrast, the uptake of 3-*O*-[methyl-^{3}H]D-glucose was not affected by Na^+. The steady-state uptake of 3-*O*-[methyl-^{3}H]D-glucose by BeWo cells was 6 μl/mg protein. The uptake of 3-*O*-[methyl-^{3}H]D-glucose by BeWo cells was reduced in the presence of 1 mM phloretin (Fig. 1B).

3.2. The transcellular transport of [^{3}H]valproic acid across the BeWo monolayer

The time course of [^{3}H]valproic acid transport across the BeWo monolayer is shown in Fig. 2. As clearly shown, the apical-to-basolateral flux was greater than the opposite flux. The apical-to-basolateral and basolateral-to-apical permeability coefficients were 4.25 ± 0.231 and 1.89 ± 0.173 μl/mg protein/min, respectively. These values were significantly higher than those of [^{14}C]mannitol (0.73 ± 0.03 and 0.77 ± 0.01, respectively).

Table 1
Concentration dependence of initial uptake of valproic acid at pH 5.5, 6.5 and 7.4

pH	Parameters			
	J_{max} (nmol/mg protein/10 s)	K_t (mM)	k_d (μl/mg protein/10 s)	J_{max}/K_t (μl/mg protein/10 s)
5.5	260.06 ± 3.03	4.07 ± 0.17	0.914 ± 0.04	63.9
6.5	203.40 ± 3.30	7.29 ± 0.38	0.893 ± 0.04	26.4
7.4	108.50 ± 1.05	11.4 ± 0.23	0.842 ± 0.16	9.52

The kinetic parameters were obtained by fitting the data to Eq. (2). Each point represents the mean ± S.E.M. of four experiments.

3.3. Temperature- and pH-dependent uptake of [^{3}H]valproic acid

We examined the time course of [^{3}H]valproic acid uptake by BeWo cells. The uptake kinetics were linear up to 15 s at pH 7.4, 6.5 and 5.5 (Fig. 3A). The initial uptake rates at pH 6.5 and pH 5.5 were 2.0- and 6.7-fold higher than that at pH 7.4. The uptake of [^{3}H]valproic acid at each pH was significantly reduced and became equal to the cellular volume at 4°C (Fig. 3B). The coefficient for [^{3}H]valproic acid uptake increased markedly when the external medium was acidic (Fig. 3C). In the presence of

Table 2
Inhibitory effects of several fatty acids on the uptake of [^{3}H]valproic acid into BeWo cells

Compounds	Concentration (mM)	Cell/medium ratio (% of control)
Acetic acid	1	68.7 ± 5.73[a]
	20	29.8 ± 2.13[a]
Lactic acid	1	60.2 ± 2.01[a]
	20	40.3 ± 0.87[a]
Propanoic acid	1	48.4 ± 2.58[a]
	20	23.0 ± 0.96[a]
Butyric acid	1	47.1 ± 1.93[a]
	20	28.7 ± 1.10[a]
Hexanoic acid	1	40.9 ± 2.48[a]
	20	11.5 ± 0.33[a]
Octanoic acid	1	35.5 ± 2.22[a]
	20	12.7 ± 1.74[a]
Pyruvic acid	1	107.4 ± 3.80
	20	100.5 ± 4.55
Stearic acid	1	105.7 ± 11.8
	20	92.6 ± 7.72
Arachidonic acid	1	95.4 ± 1.44
	20	103.1 ± 2.33
Lignoceric acid	1	109.7 ± 4.35
	20	99.7 ± 3.66

The amount of 500 μM [^{3}H]valproic acid taken up was measured at 37°C for 10 s by incubating BeWo cells in uptake buffer (pH 6.5) with each reagent. Each value represents the mean ± S.E.M. of four experiments. The control value in experiments was 14.2 ± 0.334 (μl/mg protein/10 s) and 14.2 ± 0.245 (added 0.1% albumin).

[a] Significant differences from control were identified by using ANOVA followed by Duncan's test ($P < 0.05$).

10 mM unlabeled valproic acid, the uptake of [^{3}H]valproic acid was significantly reduced at all pH investigated, and the difference between the extent of inhibition induced by unlabeled valproic acid was greater at acidic pH (Fig. 3C).

3.4. Effect of FCCP and metabolic inhibitor on the uptake of [^{3}H]valproic acid by BeWo cells

In the presence of 50 μM FCCP, a well-known protonophore, the uptake of [^{3}H]valproic acid was significantly reduced at pH 6.5 (Fig. 4A). The initial rate of [^{3}H]valproic acid uptake was 0.005 μl/mg protein/s in the presence of FCCP compared to the control value of 0.90 μl/mg protein/s. In the presence of 10 mM sodium azide, a metabolic inhibitor, the initial rate of [^{3}H]valproic acid uptake was significantly reduced to 0.024 μl/mg protein/s (control value; 0.807 μl/mg protein/s) (Fig. 4B).

3.5. The concentration-dependent uptake of [^{3}H]valproic acid by BeWo cells at pH 7.4, 6.5 and 5.5

Fig. 5 shows the Eadie–Hofstee plot of the uptake of valproic acid at pH 7.4 (A), 6.5 (B) and 5.5 (C). Eq. (2) suitably explained the characteristics of these data sets at each pH. The kinetic parameters obtained from Eq. (2) are shown in Table 1. The K_t value became smaller with a decrease in pH.

3.6. Inhibitory effects of fatty acids on the uptake of [^{3}H]valproic acid

Table 2 shows the effects of fatty acids on the uptake of [^{3}H]valproic acid by BeWo cells. Short-chain fatty acids, such as acetic acid, lactic acid, propanoic acid and butyric acid, or medium-chain fatty acids, such as hexanoic acid and octanoic acid, potently inhibited the uptake of [^{3}H]valproic acid. In contrast, long-chain fatty acids, such as arachidic acid and lignoceric acid, as well as pyruvic acid, had no effect. As shown in Table 3, PAH, DIDS, salicylic acid and furosemide, inhibitors of the anion exchange system, significantly inhibited the uptake of [^{3}H]valproic acid.

Table 3
Inhibitory effects of several compounds on the uptake of [^{3}H]valproic acid into BeWo cells

Compounds	Concentration (mM)	Cell/medium ratio (% of control)
PAH	1	66.6 ± 3.20[a]
	20	69.9 ± 2.38[a]
DIDS	1	35.9 ± 1.15[a]
	20	17.8 ± 0.82[a]
Furosemide	1	41.1 ± 1.12[a]
	20	28.5 ± 2.41[a]
Salicylic acid	1	42.3 ± 5.28[a]
	20	38.1 ± 5.27[a]

The amount of 500 μM [^{3}H]valproic acid taken up was measured at 37°C for 10 s by incubating BeWo cells in uptake buffer (pH 6.5) with each reagent. Each value represents the mean ± S.E.M. of four experiments. The control value in experiments was 14.2 ± 0.334 (μl/mg protein/10 s).

[a] Significant differences from control were identified by using ANOVA followed by Duncan's test ($P < 0.05$).

4. Discussion

This study was designed to elucidate the mechanism of valproic acid transport across the blood–placental barrier in detail, especially at the brush-border membrane facing the maternal circulation, by means of transcellular transport and uptake experiments with human placental choriocarcinoma epithelial cells (BeWo cells) in culture.

In the present study, the uptake of [^{3}H]L-alanine by BeWo cells (Fig. 1A) was shown to be Na^+-dependent, which is consistent with previous reports (Furesz et al., 1993; Moe et al., 1994; Moe, 1995; Ramamoorthy et al., 1992). In contrast, the uptake of 3-*O*-[methyl-^{3}H]D-glucose by BeWo cells was not affected by sodium ions. The steady-state uptake of 3-*O*-[methyl-^{3}H]D-glucose by BeWo cells was 6 μl/mg protein and was reduced in the presence of 1 mM phloretin (Fig. 1B). It has been reported that GLUT 1 is expressed both on the brush-border and the basal membrane of trophoblast cells (Bissonnette, 1981; Reiid and Boyd, 1994; Hahn and Desoye, 1996). Therefore, these results suggest that BeWo cells have the characteristics of normal placental trophoblasts. Indeed, BeWo cells are reported to have properties similar to those of normal trophoblasts with respect to morphology, biochemical marker enzymes and hormone secretion (Liu et al., 1997). Therefore, BeWo cells are considered to be suitable to elucidate the mechanism of drug transport across the blood–placental barrier.

The permeability coefficient of [^{3}H]valproic acid from the apical-to-basolateral side was significantly greater than that from the basolateral-to-apical side (Fig. 2), suggesting a unidirectional transport from the maternal to the fetal circulation. Hence, to elucidate the uptake system across the brush-border membrane, the first step in the transfer of the drug across the blood–placental barrier, we conducted in vitro uptake experiments under various conditions. In BeWo cells, the temperature-dependent uptake of [^{3}H]valproic acid was enhanced under acidic pH (Fig. 3A,B). The uptake coefficient of [^{3}H]valproic acid was significantly increased with a decrease in pH of the external medium (Fig. 3C). In the presence of 10 mM unlabeled valproic acid, uptake was reduced as compared with that in the presence of 500 μM valproic acid at pH 5.5–8.5 (Fig. 3C), and the extent of the reduction induced by 500 μM valproic acid was greater at acidic pH. The initial rate of [^{3}H]valproic acid uptake by BeWo cells was significantly reduced in the presence of metabolic inhibitor (Fig. 4A). These results indicate that the pH-dependent uptake of [^{3}H]valproic acid was associated with not merely the aug-

mentation of passive diffusion but also with an increase in active transport. In the presence of FCCP, a protonophore, the uptake of [^{3}H]valproic acid was significantly reduced (Fig. 4B), suggesting that the uptake of valproic acid on the brush-border membrane of BeWo cells is associated with a proton-coupled active transport system. It has been reported that there is a Na^+/H^+ exchanger on the brush-border membrane of trophoblast cells (Silva et al., 1997; Mahendran et al., 1994). Therefore, the H^+-gradient generated by the Na^+/H^+ exchanger may be the driving force of this proton-coupled active transporter in the placenta. In the kinetic analysis, the Eadie–Hofstee plot for the uptake of valproic acid at pH 7.4, 6.5 and 5.5 identified two uptake components, i.e., saturable and non-saturable components (Fig. 5). Kinetic parameters obtained from Eq. (2) are shown in Table 1. The affinity of the transporter was reduced at acidic pH, supporting the fact that the saturable transporter of valproic acid is proton-coupled. These kinetic characteristics share certain similarities with the proton-linked monocarboxylate transporter (MCT) reported previously, suggesting that the uptake of valproic acid in BeWo cells is conceivably mediated, at least in part, by MCT. Several members of MCT family, which selectively transport monocarboxylic compounds such as lactic acid, were recently cloned (Halestrap and Price, 1999; Price et al., 1998), and the expression of mRNA for the MCT family (except MCT2) was reported in the placenta (Price et al., 1998).

Recently, Utoguchi and Audus (2000) reported that valproic acid is actively transported across BeWo cells by a proton-linked transporter. However, they did not examine the pH-dependence or the effects of various inhibitors, so that detailed characteristics of valproic acid-transport across the placenta remain unknown. Therefore, we first scrutinized the effects of various inhibitors on the uptake of [^{3}H]valproic acid. As shown in Table 2, the uptake of [^{3}H]valproic acid was inhibited by short-chain fatty acids, such as acetic acid, lactic acid, propanoic acid and butyric acid, and medium-chain fatty acids, hexanoic acid and octanoic acid, while pyruvic acid and long-chain fatty acids, such as stearic acid, arachidic acid and lignoceric acid, did not affect the uptake of [^{3}H]valproic acid. Several specific transport systems for valproic acid have already been reported in the blood–brain barrier and kidney (Adkison and Shen, 1995; Adkinon et al., 1996; Naora and Shen, 1995; Ullrich et al., 1987; Ullrich, 1994). In rat brain, the translocation of [^{3}H]valproic acid was reduced by some short- and medium-chain fatty acids in a concentration-dependent manner, but was not affected by long-chain fatty acids (Adkison and Shen, 1995; Adkison et al., 1996; Naora and Shen, 1995). Therefore, the transport system for valproic acid found in the placenta in this study has characteristics similar to that in the blood–brain barrier. In the intestinal brush-border membrane, monocarboxylic acids are transported by at least two independent transporters, a proton-coupled transporter for most monocarboxylic acids and an anion antiporter for several anionic reagents, and the transport system for short-chain fatty acids is different from that for medium-chain fatty acids (Tamai et al., 1997). However, the uptake of valproic acid was equally inhibited by both short- and medium-chain fatty acids in BeWo cells, and multiple components for the uptake of valproic acid were not identified in this study.

Furthermore, DIDS, furosemide and salicylic acid significantly inhibited the uptake of [^{3}H]valproic acid (Table 3). These results are not consistent with the previous report by Utoguchi and Audus (2000). DIDS and salicylic acid inhibit the MCTs and anion exchange system (Poole and Halestrap, 1991), while furosemide acts as an inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter. Some monocarboxylic acids are likely to be transported via members of the anion exchanger family (Yabuuchi et al., 1998). The brush-border membrane of placental trophoblast cells is known to contain the Cl^-/HCO_3^- exchanger, anion exchanger-1 (AE-1) (Doughty et al., 1998; Powell et al., 1998). Although the inhibitory effects of DIDS, salicylic acid or furosemide on the uptake of [^{3}H]valproic acid might be possibly due to a decrease in intracellular Cl^- resulting from an inhibition of Cl^- influx, the contribution of these anion exchange systems still needs to be determined. Although it is as yet unknown whether valproic acid is a substrate for anion exchange systems or not, an anion exchanger may contribute to the uptake of valproic acid in BeWo cells.

Recently, mRNA of human organic anion transporter (hOAT1) was detected in the placenta by Northern blot analysis (Hosoyamada et al., 1999; Sekine et al., 1997). In this study, the uptake of valproic acid was inhibited up to 30% by PAH, a typical substrate for OAT. Therefore, the contribution of OAT to the transport of valproic acid is worth mentioning. However, in *Xenopus laevis* oocytes, OAT1 is known to act independently of sodium (Hosoyamada et al., 1999) and its affinity is much higher, with a K_t value of 9.3 μM, so that the contribution of OAT to the transport of valproic acid may be limited in the placenta.

In conclusion, our results suggest that valproic acid is transported *via* a carrier-mediated system on the brush-border membrane of placental trophoblast cells. This transport system may include a proton-coupled monocarboxylic acid transporter (MCT). Furthermore, the significant inhibition of uptake by inhibitors of the anion exchanger, such as DIDS, salicylic acid and furosemide, suggests that valproic acid may be transported by multiple transporters. These active transport systems may lead to a high rate of valproic acid transport to the fetus across the blood–placental barrier.

References

Adkison, K.D.K., Shen, D.D., 1995. Uptake of valproic acid into rat brain is mediated by a medium-chain fatty acid transporter. J. Pharmacol. Exp. Ther. 276, 1189–1200.

Adkison, K.D.K., Powers, K.M., Artru, A.A., Shen, D.D., 1996. Effect of *para*-aminohippurate on the efflux of valproic acid from the central nervous system of the rabbit. Epilepsy Res. 23, 95–104.

Bissonnette, J.M., 1981. Placental transport of carbohydrates. Mead Johnson Symp. Perinat. Dev. Med. 18, 21–23.

Doughty, I.M., Jocelyn, D.G., Powell, T.L., Jansson, T., Sibley, C.P., 1998. Chloride transport across syncytiotrophoblast microvillous membrane of first trimester human placenta. Pediatr. Res. 44, 226–232.

Furesz, T.C., Smith, C.H., Moe, A.J., 1993. ASC system activity is altered by development of cell polarity in trophoblast from human placenta. Am. J. Physiol. 265, C212–C217.

Grassl, S.M., 1998. Thiamin transport in placental brush border membrane vesicles. Biochim. Biophys. Acta 1371, 213–222.

Hahn, T., Desoye, G., 1996. Ontogeny of glucose transport systems in the placenta and its progenitor tissues. Early Pregnancy 2, 168–182.

Halestrap, A.P., Price, N.T., 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem. J. 343, 281–299.

Hay Jr., W.W., 1994. Placental transport of nutrients to the fetus. Horm. Res. 42, 215–222.

Hosoyamada, M., Sekine, T., Kanai, Y., Endou, H., 1999. Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. Am. J. Physiol. 276, F122–F128.

Hu, Z.-Q., Henderson, G.I., Mock, D.M., Schenker, S., 1994. Biotin uptake by basolateral membrane vesicles of human placenta: normal characteristics and role of ethanol. Proc. Soc. Exp. Biol. Med. 206, 404–408.

Ishizaki, T., Yokochi, K., Chiba, K., Tabuchi, T., Wagatsuma, T., 1981. Placental transfer of anticonvulsants (phenobarbital, phenytoin, valproic acid) and the elimination from neonates. Pediatr. Pharmacol. 1, 291–303.

Johannessen, S.I., 1992. Pharmacokinetics of valproic in pregnancy: mother–foetus–newborn. Pharm. Weekbl. Sci. Ed. 14, 114–117.

Liu, F., Michael, S.J., Kenneth, A.L., 1997. Permeability properties of monolayers of the human trophoblast cell line BeWo. Am. J. Physiol. 273, C1596–C1604.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malone, F.D., D'Alton, M.E., 1997. Drugs in pregnancy: anticonvulsants. Semin. Perinatol. 21, 114–123.

Mahendran, D., Byrne, S., Donnai, P., D'Souza, S.W., Glazier, J.D., Jones, C.J.P., Sibley, C.P., 1994. Na^+ transport, H^+ concentration dissipation, and system A amino acid transporter activity in purified microvillous plasma membrane isolated from first trimester human placenta: comparison with the term microvillous membrane. Am. J. Obstet. Gynecol. 171, 1534–1540.

Moe, A.J., 1995. Placental amino acid transport. Am. J. Physiol. 268, C1321–C1331.

Moe, A.J., Furesz, T.C., Smith, C.H., 1994. Functional characterization of L-alanine transport in placental choriocarcinoma cell line (BeWo). Placenta 15, 797–802.

Naora, K., Shen, D.D., 1995. Mechanism of valproic acid uptake by isolated rat brain microvessels. Epilepsy Res. 22, 97–106.

Pacifici, G.M., Nottoli, R., 1995. Placental transfer of drugs administered to the mother. Clin. Pharmacokinet. Concept. 28, 235–269.

Pattilo, R.A., Gey, G.O., Delfs, E., Mattingly, R.F., 1986. In vitro identification of the trophoblast stem cell of the human villous placenta. Am. J. Obstet. Gynecol. 100, 582–588.

Powell, T.L., Lundquist, C., Doughty, I.M., Glazier, J.D., Jansson, T., 1998. Mechanisms of chloride transport across the syncytiotrophoblast basal membrane in human placenta. Placenta 19, 315–321.

Poole, C.R., Halestrap, A.P., 1991. Reversible and irreversible inhibition, by stilbendisulphonates, of lactate transport into rat erythrocytes. Biochem. J. 275, 307–312.

Prasad, P.D., Wang, H., Kekuda, R., Fujita, T., Fei, Y., Devoe, L.D., Leibach, F.H., Ganapathy, V., 1998. Cloning and functional expression of a cDNA encoding a mammalian sodium-dependent vitamin transporter mediating the uptake of pantothenate, biotin, and lipoate. J. Biol. Chem. 273, 7501–7506.

Price, N.T., Jakson, V.N., Halestrap, A.P., 1998. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. Biochem. J. 329, 321–328.

Ramamoorthy, S., Leibach, F.H., Mahesh, V.B., Ganapathy, V., 1992. Selective impairment of taurine transport by cyclosporin A in a human placental cell line. Pediatr. Res. 32, 125–127.

Reiid, N.A., Boyd, C.A.R., 1994. Further evidence for the presence of two facilitative glucose transporter isoforms in the brush border membrane of the syncytiotrophoblast of the human full term placenta. Biochem. Soc. Trans. 22, 267S.

Schenker, S., Johnson, R.F., Mahuren, J.D., Henderson, G.I., Coburn, S.P., 1992. Human placental vitamin B6 (pyridoxal) transport: normal characteristics and effects of ethanol. Am. J. Physiol. 262, R966–R974.

Sekine, T., Watanabe, N., Hosoyamada, M., Kanai, Y., Endou, H., 1997. Expression cloning and characterization of a novel multispecific organic anion transporter. J. Biol. Chem. 272, 18526–18529.

Silva, N.L.C.L., Wang, H., Harris, C.V., Singh, D., Fliegel, L., 1997. Characterization of the Na^+/H^+ exchanger in human choriocarcinoma (BeWo) cells. Pflugers Arch. Eur. J. Physiol. 433, 792–802.

Stulc, J., 1997. Placental transfer of inorganic ions and water. Physiol. Rev. 77, 805–836.

Tamai, I., Takanaga, H., Maeda, H., Yabuuchi, H., Sai, Y., Suzuki, Y., Tsuji, A., 1997. Intestinal brush-border membrane transport of monocarboxylic acids by proton-coupled transport and anion antiport mechanisms. J. Pharm. Pharmacol. 49, 108–112.

Ullrich, K.J., 1994. Specificity of transporters for 'organic anions' and 'organic cations' in the kidney. Biochim. Biophys. Acta 1197, 45–62.

Ullrich, K.J., Fritzsch, R.G., Kloss, S., 1987. Contraluminal *para*-aminohippurate transport in the proximal tubule of the rat kidney: III. Specificity: monocarboxylic acids. Pflugers Arch. Eur. J. Physiol. 409, 547–554.

Utoguchi, N., Audus, K.L., 2000. Carrier-mediated transport of valproic acid in BeWo cells, a human trophoblast cell line. Int. J. Pharm. 195, 115–124.

Van der Aa, E.M., Peereboom-Stegeman, J.H.J.C., Noordhoek, J., Gribnau, F.W.J., Russel, G.M., 1998. Mechanisms of drug transfer across the human placenta. Pharm. World Sci. 20, 139–148.

Yabuuchi, H., Tamai, I., Sai, Y., Tsuji, A., 1998. Possible role of anion exchanger AE2 as the intestinal monocarboxylic acid/anion antiporter. Pharm. Res. 15, 411–416.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharmacobio.-Dyn. 4, 879–885.

Yerby, M.S., 1997. Teratogenic effects of antiepileptic drugs: what do we advise patients? Epilepsia 38, 957–958.