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Research paper

Transport of valproate at intestinal epithelial (Caco-2) and brain endothelial (RBE4) cells: Mechanism and substrate specificity

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ABSTRACT

To reach its target cells, the antiepileptic drug valproate has to cross both the intestinal epithelial barrier and the blood–brain barrier in intact form as well as in sufficient amounts. This study was performed to characterize the epithelial transport of valproate at intestinal (Caco-2) and at blood–brain barrier (RBE4) cells. At both cell types, uptake of [^{3}H]valproate was independent of inwardly directed Na^+, Ca^{2+}, Mg^{2+}, K^+ or Cl^- gradients. Uptake was, however, strongly stimulated by an inwardly directed H^+ gradient. The cells accumulated valproate against a concentration gradient and the uptake rate of valproate was saturable with K_t values of 0.6 and 0.8 mM. At Caco-2 cell monolayers, the total apical-to-basolateral flux of [^{3}H]valproate exceeded the basolateral-to-apical flux 14-fold. Various monocarboxylic acids like salicylate, benzoate, acetate, propionate, butyrate, hexanoate, diclofenac and ibuprofen inhibited [^{3}H]valproate uptake at both cell types. Lactate and pyruvate inhibited valproate uptake at RBE4 cells but not at Caco-2 cells. We conclude that valproate is accumulated in intestinal cells against a concentration gradient by the activity of a specific H^+-dependent DIDS-insensitive transport system for monocarboxylates not identical with monocarboxylate transporter 1 (MCT1). The passage of valproate across the blood–brain barrier is very likely mediated by MCT1.

1. Introduction

Valproate is widely used for the treatment of epilepsy and bipolar disorder as well as for migraine prophylaxis [1]. The drug increases the brain level of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) by various mechanisms such as decreasing GABA catabolism, causing GABA-mimetic effects on postsynaptic receptors and reducing conductance for Na^+ and K^+ ions [2,3]. Valproate interferes with multiple regulatory and signaling mechanisms, enhances activator protein 1-binding and acts as inhibitor of histone deacetylases [4–6].

In pharmacotherapeutic routine, valproate is administered orally at a dose of 20–30 mg/kg [7]. To reach its target cells, the drug has to cross both the intestinal epithelial barrier and the blood–brain barrier in intact form as well as in sufficient amounts. The oral bioavailability of valproate is close to 100%. Cato and coworkers examined the active vs. passive components of valproate absorption in everted intestinal sacs prepared from different segments of rat intestine [8]. Data were consistent with active transport of valproate in the jejunum and ileum. Tori et al. using Caco-2 cells have shown that apical-to-basolateral transepithelial valproate transport is inhibited by carbapenem antibiotics [9]. There is evidence that valproate interacts with monocarboxylic acid transporters (MCT). In several studies the group of A. Tsuji demonstrated H^+-cotransport of compounds such as acetic acid, benzoic acid, salicylate or mevalonic acid that was inhibited by valproate [10–13]. These endogenous metabolically important monocarboxylates are transported by members of the MCT family, mainly MCT1–4 [14,15]. At the human intestine, expression of at least MCT1, 3, 4 and 5 has been shown [14–16]. Inhibition of uptake of labeled MCT substrates by valproate, however, does not mean that valproate itself is transported by the respective MCT. Similarly, it has been shown that valproate inhibits the transport of estrone-3-sulfate by the organic anion transporting polypeptide OATP2B1 in Caco-2 cells [17] but this result does not allow the conclusion that valproate is a substrate of OATP2B1 [18]. Valproate also significantly decreased the H^+-dependent intestinal uptake of nateglinide mediated by a system distinct from MCT1, again without being necessarily a substrate [19,20].

After absorption into the blood circulation, to reach the target neurons in the brain, valproate has to cross the blood–brain

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barrier. For therapeutic effects, the blood serum concentration of valproate is maintained between 60 and 120 mg/l, i.e. between 0.36 and 0.72 mM [7]. It has long been believed that lipophilic drugs like valproate cross membranes in a passive non-saturable manner according to the pH-partition theory. However, just as for the intestinal epithelium, several studies indicated the participation of saturable, active transport systems for influx and efflux across the blood–brain barrier [21–23]. The systems under discussion for valproate influx or efflux, respectively, are anion exchange systems different from MCT, organic anion transporters (OAT [24]), members of the multidrug resistance protein (MRP) family other than P-glycoprotein, MRP1 and MRP2 [22,25], a medium-chain fatty acid transporter that does not interact with short monocarboxylic acids [26], members of the MCT family [27] and other systems. The expression of MCT1 and MCT2 at brain endothelial cells has been postulated and an inhibitory effect of valproate on the blood–brain barrier uptake of acetate, salicylate, lactate, pyruvate, butyrate and others suggesting a direct or indirect interaction with MCT systems has been reported [3,28]. Comparable results have been obtained at other important epithelial barriers and cell types such as the blood–cerebrospinal fluid barrier were involvement of a carrier for organic anions has been described [29]. At the human placenta, a H^+-dependent, saturable, and asymmetric transport system, presumed to be a MCT, is predominantly responsible for valproate uptake [30,31]. At primary cultures of rat astroglial cells valproate interferes with the uptake of L-glutamate, L-aspartate and GABA [2,32,33].

Only in a very few studies valproate uptake by carriers after heterologous expression has been investigated. For example, Yabuuchi et al. [34] reported valproate transport activity of the anion exchanger 2 expressed in HEK293 cells. However, even if transport of valproate by a specific system has been measured, the result does not allow the conclusion that this particular system is the primary and pharmacologically relevant valproate transporter. For that, the transport mechanism and direction, the substrate specificity of valproate uptake and the substrate saturation kinetics in relation to the drugs therapeutic concentration have to be determined.

2. Materials and methods

2.1. Materials

The cell line Caco-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The RBE4 cell line was kindly provided by Françoise Roux, (INSERM, Paris, France). [4,5-^{3}H]Valproate ([^{3}H]valproate, specific activity 55 Ci/mmol) was obtained from Moravek Biochemicals (Brea, USA) and [7-^{14}C]salicylate ([^{14}C]salicylate, specific activity 55.5 Ci/mmol) from NEN (Boston, USA). Cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany) and PAA Laboratories GmbH (Cölbe, Germany). Carbamazepine was purchased from ICN Biomedicals (Ohio, USA). Nateglinide was a gift of Ajinomoto Co., Inc. (Tokyo, Japan). Other chemicals were obtained from Sigma (Taufkirchen, Germany).

2.2. Cell culture

Caco-2 cells were routinely cultured (passages 34–90) in 75-cm^2 culture flasks (Greiner, Frickenhausen, Germany) with minimum essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution and gentamicin (45 μg/ml) [35,36]. Cells grown to 80% confluence were released by trypsinization and subcultured in 35 mm disposable petri dishes (Sarstedt, Nümbrecht, Germany) at a density of $0.8 \cdot 10^6$ cells/dish. The cultures reached confluence within 24 h, uptake was measured 7 days after seeding. Caco-2 cells were also cultured on permeable polycarbonate Transwell® cell culture inserts (diameter 24.5 mm, pore size 3 μm, Corning Life Sciences, Schiphol-Rijk, The Netherlands) with a cell density of 42,500 cells/cm^2 for 19–24 days as described [35,36]. At this stage the Caco-2 cell monolayers in this study displayed a transepithelial electrical resistance of 326 ± 9 Ω cm^2. The medium was replaced 24 h after subculturing, every 2 days and the day before the uptake experiment.

Culture medium for RBE4 cells was α-MEM/Ham's F10 (1:1), supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml), geneticin (300 μg/ml) and basic fibroblast growth factor (1 ng/ml). Cells grown to 100% confluence were released by trypsinization and subcultured in 35 mm disposable petri dishes. With a starting cell density of $0.5 \cdot 10^6$ cells/dish the cells reached confluence after 24 h. The medium was replaced every day. Uptake was measured 4 days after seeding.

2.3. Transport measurements

The uptake of [^{3}H]valproate and [^{14}C]salicylate was measured at room temperature [35–37]. Monolayers were rinsed two times with buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM MES/Tris (pH 6.0), 25 mM Hepes/Tris (pH 7.5) or 25 mM Tris/Hepes (pH 8.5). For Na^+-free buffer, NaCl was replaced with 140 mM choline chloride. For apparently K^+-, Ca^{2+}- or Mg^{2+}-free buffer, KCl, $CaCl_2$ or $MgSO_4$ were replaced by equimolar NaCl. K^+-rich buffer contained 5.4 mM NaCl and 140 mM KCl. For Cl^--free buffer, NaCl, KCl and $CaCl_2$ were replaced by the respective gluconate salts. To initiate uptake, 1 ml buffer containing 1 nM [^{3}H]valproate or 3 nM [^{14}C]salicylate, respectively, and unlabeled compounds at increasing concentrations were added to each dish. After incubation for the desired time (0–10 min) buffer was removed and monolayers were quickly washed four times with ice-cold uptake buffer. Cells were solubilized and prepared for liquid scintillation counting. The protein content was measured according to the method of Bradford.

Transepithelial flux of [^{3}H]valproate across Caco-2 cell monolayers cultured on permeable filters was measured in both directions at 37 °C at pH 6.0 at the apical (1.5 ml) and pH 7.5 at the basolateral side (2.6 ml [35–37]). After measuring the transepithelial electrical resistance the inserts were washed with buffer two times. Flux experiments were started by adding buffer containing [^{3}H]valproate (1 nM) with or without 10 mM unlabeled valproate to the donor side. At given times samples were taken from the receiver compartment and replaced with fresh buffer. After 2 h, the filters were washed, cut out of the plastic insert and also prepared for liquid scintillation analysis.

2.4. Data analysis

In general, each data point was determined at least in triplicate for each experiment. Data are presented as mean ± SE. Statistical analyses were done with the *U*-test by Mann and Whitney. The kinetic constants were calculated by non-linear regression of the Michaelis–Menten plot and confirmed by linear regression of the Eadie–Hofstee plot [35,37]. The calculated parameters are shown with their SE. Inhibition constants (K_i) were calculated from the IC_{50} values (i.e. the concentration of the unlabeled compound necessary to inhibit 50% of specific [^{3}H]valproate uptake) using the K_t values of 0.6 mM (Caco-2) and 0.8 mM (RBE4), respectively, obtained in this study.

3. Results

Uptake of [^{3}H]valproate (1 nM, pH 6.0) in Caco-2 cells reached a maximum of 153 fmol per 10 min per mg of protein after a cell culture time of 6–8 days (data not shown). Therefore, for the following studies a culture period of 7 days was chosen. RBE4 cells were cultured for 4 days when [^{3}H]valproate uptake (1 nM, pH 6.0) was 390 fmol per 2 min per mg of protein. We then investigated the dependence of [^{3}H]valproate uptake on inwardly directed ion gradients. Replacing extracellular NaCl with choline chloride had no effect on [^{3}H]valproate uptake (Fig. 1A and B). Hence, [^{3}H]valproate uptake is a Na^+-independent process. In contrast, [^{3}H]valproate uptake was highly affected by the outside pH. In Caco-2 cells, uptake was 20-fold higher at an outside pH of 6.0 compared to outside pH 8.5 (Fig. 1A). At RBE4 cells the pH effect was even stronger with a 32-fold stimulation by pH 6.0 (Fig. 1B). This pH stimulation was found both in the presence and in the absence of Na^+ (140 mM).

The uptake of [^{3}H]valproate was highly saturable. Unlabeled valproate at an excess amount of 10 mM decreased uptake of labeled valproate measured at pH 6.0 by 89% in Caco-2 cells and by 91% in RBE4 cells, respectively (Fig. 1). In order to elucidate whether the strong stimulation of [^{3}H]valproate uptake by H^+ is due to the outside acidic pH per se or due to the inwardly directed pH gradient, we investigated the effect of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). FCCP dissipates proton gradients across cell membranes. When present in the uptake buffer, FCCP (50 μM) reduced [^{3}H]valproate uptake by 68% in Caco-2 (Fig. 1A, inset) and by 79% in RBE4 cells (Fig. 1B, inset). Therefore, it is the pH gradient across the membrane that drives [^{3}H]valproate uptake. With regard to other ion gradients, we found that the omission of Ca^{2+}, Mg^{2+}, Cl^- and K^+ from the uptake buffer as well as increasing the extracellular K^+ concentration to 140 mM had no effect on [^{3}H]valproate uptake in both cells (data not shown). Next, before determination of the kinetic parameters, the time course of [^{3}H]valproate uptake had to be recorded (Fig. 2). Time-dependent uptake saturated rapidly in both cell types. For kinetic experiments at Caco-2 cells, a 2-min uptake period and for those at RBE4 cells a 1-min uptake time was chosen. After 5 min, [^{3}H]valproate uptake reached a plateau of 262 fmol/mg of protein in Caco-2 cells and 474 fmol/mg of protein in RBE4 cells. Assuming an intracellular accessible cell volume of about 10 μl/mg of protein, the intracellular concentration reached 26 nM in Caco-2 cells and 47 nM in RBE4 cells. Considering the extracellular [^{3}H]valproate concentration of 1 nM during the uptake measurements, the cells accumulate [^{3}H]valproate uphill approximately 25- and 50-fold, respectively.

To determine the kinetic parameters of valproate uptake, Caco-2 cells were incubated for 2 min at pH 6.0 with [^{3}H]valproate (1 nM) at increasing valproate concentrations (0–10 mM). Fig. 3A shows the total valproate uptake in Caco-2 cells before correction for the non-mediated transport components. This linear non-saturable component was quantified by measuring [^{3}H]valproate uptake (1 nM) in the presence of 31.6 mM unlabeled valproate. It was subtracted from total uptake before transformation of the data into the Eadie–Hofstee plot (Fig. 3A, inset). This component was 8.3% of the total [^{3}H]valproate uptake per 2 min. Kinetic analysis performed by non-linear regression of the carrier mediated valproate uptake revealed an apparent affinity constant (K_t) of 0.6 ± 0.2 mM and a maximal velocity (V_{max}) of 146.1 ± 12.6 nmol/2 min per mg of protein.

Likewise, the kinetic parameters of valproate uptake were determined in RBE4 cells. Total uptake per 1 min is shown in Fig. 3B. At these cells, the linear non-saturable component of the total [^{3}H]valproate uptake was with 1.8% of total uptake even lower than at Caco-2 cells. The relationship between saturable uptake rate and substrate concentration was found to be hyperbolic. We obtained an apparent Michaelis constant (K_t) of 0.8 ± 0.1 mM and a maximal velocity (V_{max}) of 244.6 ± 9.5 nmol/min per mg of protein.

We then studied in detail the substrate specificity of valproate uptake. [^{3}H]Valproate uptake into Caco-2 cells could be inhibited not only by unlabeled valproate itself but also by several other

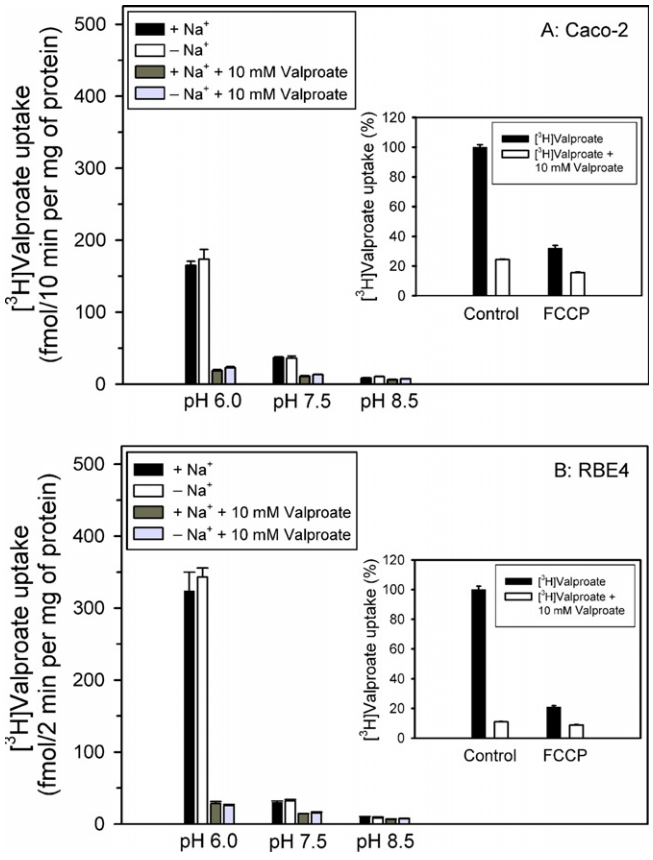

Fig. 1. Effect of extracellular pH, Na^+ and unlabeled valproate (10 mM) on [^{3}H]valproate uptake in Caco-2 (A) and RBE4 (B) cells. Uptake of [^{3}H]valproate (1 nM) was measured for 10 min (Caco-2) or 2 min (RBE4) at room temperature. Sodium chloride was iso-osmotically replaced by choline chloride. Insets: Effect of FCCP on the uptake of [^{3}H]valproate (1 nM) was measured in the absence (control) and presence of FCCP (50 μM) at pH 6.0 for 10 min (A) or 2 min (B). Values represent means ± SE, n = 3.

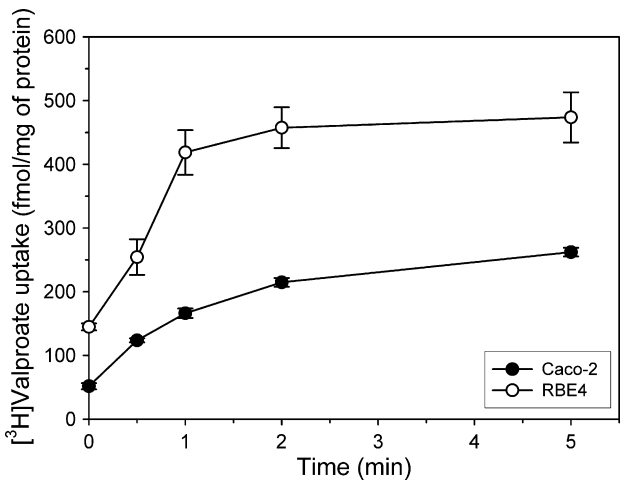

Fig. 2. Time-dependent [^{3}H]valproate uptake in Caco-2 (A) and RBE4 (B) cells. Uptake of [^{3}H]valproate (1 nM) was measured at pH 6.0 in cells cultured 6 (Caco-2) or 3 (RBE4) days post confluence. Values represent means ± SE, n = 4.

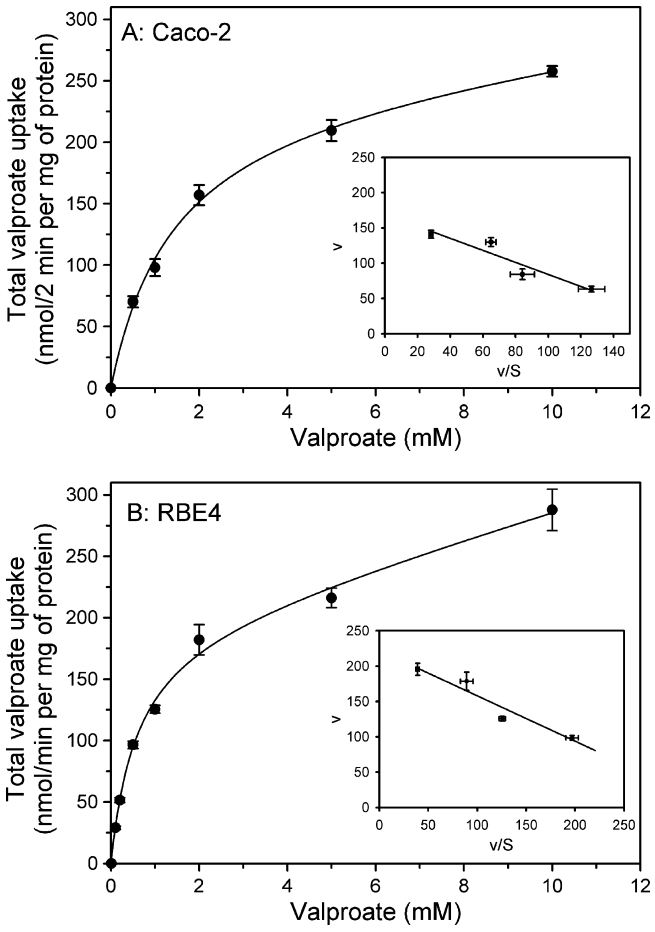

Fig. 3. Substrate saturation kinetics of valproate uptake in Caco-2 (A) and RBE4 (B) cells. Uptake of valproate was measured over a concentration range of 1 nM to 10 mM. The results represent total uptake rate values not corrected for non-saturable (linear) transport components. Inset: Eadie–Hofstee transformation of the data after subtraction of non-saturable [^{3}H]valproate uptake. *v*, uptake rate in nmol/2 min (A) and nmol/min (B) per mg of protein; *S*, concentration of valproate (mM), $n = 4$.

compounds (Table 1). Salicylate, benzoate as well as 2- and 4-aminobenzoate, the short-chain fatty acids acetate, propionate and butyrate (all 10 mM) strongly inhibited [^{3}H]valproate uptake by more than 70%. Moreover, the non-steroidal antiphlogistics diclofenac (2 mM), ibuprofen (2 mM), indomethacine (1 mM) and piroxicam (1 mM) reduced the uptake to 23–68% (Table 1). In contrast, L- and D-lactate, pyruvate, GABA and γ-hydroxybutyrate showed no significant inhibitory effects. [^{3}H]Valproate uptake was not affected by several prototype substrates of amino acid transporters, organic cation or anion transporters and several other well-known transporters, i.e. not by bumetanide, phloretin, L-triiodothyronine, *p*-aminohippurate, tetraethylammonium and *N*-methyl-4-phenylpyridinium or L-proline (Table 1). For the most effective inhibitors we performed detailed dose–response experiments using a broad concentration range from 0 to 31.6 mM to determine the apparent inhibitory constants (K_i) for [^{3}H]valproate uptake inhibition. These competition assays resulted in inhibitory constants between 1.7 mM for butyrate and 5.2 mM for acetate (Table 2).

Substrate specificity was also studied at RBE4 cells (Table 1). Valproate, benzoic acid, diclofenac and ibuprofen were found to be strong valproate uptake inhibitors. In general, the inhibition strength of a specific compound was significantly higher in RBE4 cells than in Caco-2 cells. This is most likely due to the very low unspecific transport rate of [^{3}H]valproate in RBE4. Remarkably,

Table 1
Substrate specificity of [^{3}H]valproate uptake

Compound	[^{3}H]Valproate uptake (%)	
	Caco-2	RBE4
Control	100 ± 1	100 ± 1
Valproate	15.3 ± 0.6[a]	8.1 ± 0.6[a]
L-Lactate	94.0 ± 0.9[a]	26.5 ± 5.4[a]
D-Lactate	98.3 ± 3.6[a]	41.3 ± 1.2[a]
Acetate	29.5 ± 1.1[a]	5.2 ± 0.2[a]
Propionate	18.6 ± 1.1[a]	5.7 ± 0.3[a]
Butyrate	17.1 ± 0.2[a]	6.5 ± 0.1[a]
Hexanoate	23.1 ± 0.8[a]	–
Pyruvate	98.8 ± 2.0	45.2 ± 0.9[a]
Salicylate	12.0 ± 0.3[a]	2.5 ± 0.3[a]
Benzoate	12.2 ± 1.8[a]	6.3 ± 1.6
2-Aminobenzoate	15.4 ± 0.7[a]	–
3-Aminobenzoate	76.1 ± 3.9[a]	–
4-Aminobenzoate	24.7 ± 1.6[a]	–
GABA	88.5 ± 1.3[a]	–
γ-Hydroxybutyrate	94.3 ± 1.9[a]	29.1 ± 1.7[a]
Bumetanide (2 mM)	148 ± 20	77.9 ± 4.4[a]
Probenecid (2 mM)	54.6 ± 1.9[a]	11.6 ± 1.0[a]
Nateglinide (2 mM)	55.3 ± 5.8[a]	8.6 ± 1.4[a]
Isobutyl-methyl-xanthine (2 mM)	97.0 ± 3.0[a]	84.0 ± 4.3
L-Triiodothyronine (0.05 mM)	101 ± 1	100 ± 2
L-Phenylalanine	98.0 ± 1.4	–
Nicotinate	85.7 ± 2.2[a]	38.2 ± 2.5[a]
DIDS (1 mM)	123 ± 1[a]	–
Phloretin (0.1 mM)	108 ± 5	78.1 ± 1.3[a]
Rifampicin (1 mM)	98.9 ± 11.7	–
Carbamazepine (1 mM)	92.2 ± 2.2	–
Acetaminophen (Paracetamole)	85.1 ± 12.3	–
Aminosalicylate	77.1 ± 1.2[a]	–
Metamizol	90.7 ± 4.6	–
Diclofenac (2 mM)	23.2 ± 0.7[a]	2.4 ± 0.4[a]
Ibuprofen (2 mM)	32.6 ± 0.8[a]	11.1 ± 0.2[a]
Indomethacin (1 mM)	52.2 ± 1.3[a]	–
Piroxicam (1 mM)	68.0 ± 5.6[a]	–
Baclofen (2 mM)	102 ± 10	–
L-Glutamate	100 ± 3	–
L-Aspartate	92.3 ± 7.0	89.9 ± 3.1
L-Proline	92.3 ± 0.2[a]	91.8 ± 3.4
3-OH-L-Proline	90.8 ± 6.8	–
L-Valine	97.2 ± 2.4	–
p-Aminohippurate	98.9 ± 1.6	89.9 ± 3.0
Cefadroxil	105 ± 2	97.1 ± 6.0
N-Methyl-4-phenylpyridinium	99.6 ± 3.1	88.0 ± 2.1[a]
Tetraethylammonium	101 ± 6	90.6 ± 3.1
Carnitine	97.8 ± 1.7	90.8 ± 1.2
Clonidine	108 ± 3	–
Codeine	100 ± 3	–
Verapamil	107 ± 3	89.0 ± 2.7[a]
Estrone-3-sulfate	107 ± 2	–

Uptake of [^{3}H]valproate (1 nM, pH 6.0, $t = 2$ min (Caco-2 cells) or 1 min (RBE4 cells)) was measured in the absence (control) or presence of unlabeled test compounds at 10 mM or as indicated. Data are means ± SE, ($n \geqslant 4$).

[a] Significantly different from control with $p \leqslant 0.05$.

Table 2
Inhibition constants (K_i) of different compounds for the inhibition of [^{3}H]valproate uptake

Compound	K_i (mM)	
	Caco-2	RBE4
Valproate	0.58 ± 0.2	0.81 ± 0.1
L-Lactate	>100	2.5 ± 0.2
Acetate	5.2 ± 0.1	0.8 ± 0.1
Propionate	2.2 ± 0.1	0.5 ± 0.1
Butyrate	1.7 ± 0.2	0.6 ± 0.1
Salicylate	2.2 ± 0.2	0.5 ± 0.1
Nateglinide	2.1 ± 0.1	0.4 ± 0.1

Uptake of [^{3}H]valproate (1 nM) was measured at pH 6.0 for 2 min (Caco-2) or 1 min (RBE4) in presence of unlabeled inhibitors (0–31.6 mM). Parameter is shown ± SE, $n = 4$.

and in strong contrast to Caco-2 cells, [^{3}H]valproate uptake into RBE4 was inhibited >50% by L-lactate, D-lactate, pyruvate, γ-hydroxybutyrate, probenecid and nicotinate. Moreover, a weak but significant inhibition by bumetanide was found. In competition assays K_i values between 0.4 and 0.8 mM were determined for most inhibitors. Salicylate, acetate, propionate and butyrate showed higher inhibition, i.e. lower K_i values at RBE4 cells than at Caco-2 cells. The decisive difference between valproate uptake at Caco-2 and RBE4 cells was that at RBE4 cells the prototype MCT1–4 substrate L-lactate inhibited uptake with a K_i value of 2.5 mM, whereas at Caco-2 cells no inhibition was found.

Having observed that salicylate is a potent inhibitor of [^{3}H]valproate uptake both at Caco-2 cells and at RBE4 cells, we examined whether valproate affects the uptake of salicylate. The monolayers were incubated with [^{14}C]salicylate (3 nM) for 10 min at pH 6.0 and 7.5 in the absence (control) and presence of an excess amount (10 mM) of salicylate or valproate, respectively. Uptake of [^{14}C]salicylate in Caco-2 cells and RBE4 cells was stimulated more than 10-fold by an extracellular pH of 6.0 (Fig. 4). At this pH, uptake was strongly reduced by 64–91% in the presence of excess amounts (10 mM) of unlabeled valproate or salicylate (Fig. 4).

In the next series of experiments, we studied the bidirectional transepithelial transport of [^{3}H]valproate across Caco-2 cell monolayers cultured on permeable filters for 19–24 days. We determined the net transepithelial flux of [^{3}H]valproate (1 nM) in apical-to-basolateral (J_{a-b}) direction, in basolateral-to-apical direction (J_{b-a}) and, after finishing the flux experiment after 2 h, the uptake into the cell monolayers from the apical side (J_{a-c}) and from the basolateral side (J_{b-c}). All measurements were done in the absence and presence of an excess amount of unlabeled valproate (10 mM). Flux was linear for 30 min and rapidly reached a plateau in 1–2 h. After this time, donor and acceptor were equilibrated, whether [^{3}H]valproate had been added to the apical or to the basolateral compartment. The flux rates shown in Table 3 were therefore determined by linear regression of flux data up to 30 min.

The total transepithelial flux J_{a-b} was 21.0 ± 1.2%/h/cm^2 (=291.2 ± 17 fmol/h/cm^2) and exceeded the flux of the non-transported hydrophilic space marker [^{14}C]mannitol by a factor of 150 (0.14 ± 0.03%/h/cm^2). J_{a-b} of [^{3}H]valproate was moderately reduced in the presence of 10 mM valproate to 81%, whereas the intracellular accumulation J_{a-c} of [^{3}H]valproate from the apical compartment into the cell monolayers within 2 h was strongly reduced from 0.77 ± 0.08 fmol/2 h/cm^2 to 0.26 fmol/2 h/cm^2 (Table 3).

Compared to J_{a-b}, the net transepithelial flux of [^{3}H]valproate (1 nM) in the basolateral-to-apical direction (J_{b-a}) was 14-fold lower

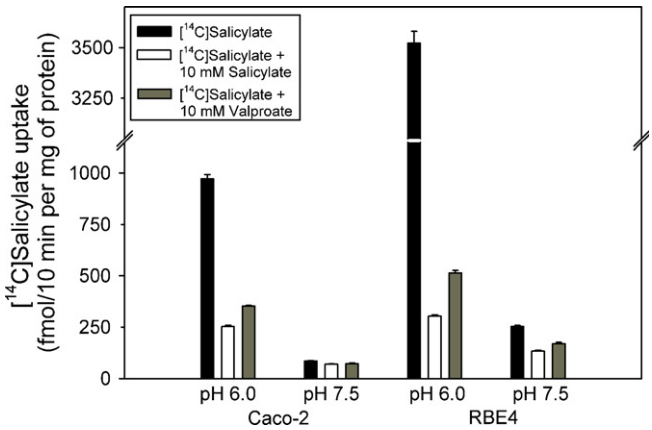

Fig. 4. Effect of unlabeled valproate and salicylate on pH-dependent [^{14}C]salicylate uptake in Caco-2 (A) and RBE4 cells (B). Uptake of [^{14}C]salicylate (3 nM) was measured for 2 min (Caco-2) or 10 min (RBE4) in the absence (control) and presence of salicylate and valproate (10 mM) at pH 6.0 and 7.5. Values represent means ± SE, n = 4.

Table 3
Bidirectional transepithelial flux and intracellular accumulation of [^{3}H]valproate at Caco-2 monolayers

	Flux		Uptake	
	fmol/h/cm^2	%	fmol/2 h/cm^2	%
J_{a-b}				
Control	291.2 ± 16.9	100	0.77 ± 0.08	100
Valproate	237.1 ± 21.4[a]	81[a]	0.26 ± 0.07[a]	33[a]
J_{b-a}				
Control	20.3 ± 0.4	7 (100)	2.0 ± 0.4	258 (100)
Valproate	20.2 ± 4.2[a]	7 (100)	0.43 ± 0.13[a]	56[a] (21)

Transepithelial flux and uptake of [^{3}H]valproate (1 nM) from apical-to-basolateral (J_{a-b}) and from basolateral-to-apical side (J_{b-a}) was measured in the absence (control) and presence of 10 mM unlabeled valproate, (n = 4).
[a] Significantly different from control with $p \leqslant 0.05$.

(20.3 ± 0.4 fmol/h/cm^2, Table 3). Moreover, J_{b-a} could not be inhibited by unlabeled valproate (10 mM) added to the same (basolateral) donor compartment. In contrast, the uptake of [^{3}H]valproate into Caco-2 cell monolayers across the basolateral cell membranes (J_{b-c}) within 2 h could be inhibited by 10 mM unlabeled valproate by 79% (Table 3).

4. Discussion

The results of the present investigation can be summarized as follows: both intestinal cells and blood–brain barrier endothelial cells accumulate valproate against a concentration gradient. Transport is saturable with Michaelis constants of 0.6 and 0.8 mM, respectively. Uptake was found independent of Na^+, Ca^{2+}, Mg^{2+}, K^+ and Cl^- gradients but strongly stimulated by an outside acidic pH. The pH gradient itself is the driving force for uptake. At Caco-2 cell monolayers, the total apical-to-basolateral flux of [^{3}H]valproate exceeds the basolateral-to-apical flux 14-fold. Interestingly, when expressed per mg of protein, at RBE4 cells we observed a 2- to 5-fold higher valproate uptake compared to Caco-2 cells. Various monocarboxylic acids like salicylate, benzoate, acetate, propionate, butyrate, hexanoate, diclofenac and ibuprofen interact with the valproate uptake system. Importantly, lactate and pyruvate were not able to inhibit intestinal valproate uptake. Valproate inhibits salicylate uptake in both cells types.

According to our study, intestinal bidirectional valproate transport is predominantly an absorptive process rather than a secretory process. This corresponds well with investigations of transepithelial nateglinide transport at Caco-2 cells that demonstrated higher transepithelial flux from apical-to-basolateral side than in opposite direction [19]. Apical-to-basolateral flux of labeled benzoate and salicylate across Caco-2 cells was found to be inhibited by acetate, salicylate, benzoate and valproate [11,12,38]. Transport of benzoate occurred predominantly in apical-to-basolateral direction [38].

Such experiments were not possible with RBE4 cells because these cells derived from endothelial cells of the rat blood–brain barrier do not form monolayers tight enough for specific transcellular flux studies on filters. In vivo, however, using the intracarotid injection technique, Cornford and coworkers [21] observed asymmetric properties of the blood–brain barrier to valproate where brain-to-blood transport exceeds blood-to-brain transport. At placental choriocarcinoma epithelial cells (BeWo) unidirectional transepithelial transport of valproate was determined that was higher in the fetal direction and stimulated by an acidic apical pH [31].

The first step, the valproate uptake into the cells, is mediated by an H^+-symporter rather than a Na^+-symporter such as the sodium-coupled monocarboxylate cotransporter SMCT1 (SLC6A8). Such a relatively specific H^+-dependent transport mechanism has already been well established for monocarboxylic acids such as lactate, pyruvate, benzoate, acetate or salicylate by many authors

[10–15,19,27]. In several of these studies, valproate was able to inhibit transport of other monocarboxylates at intestinal cells suggesting at least interaction of valproate with these systems. Transport of valproate itself cannot be concluded from inhibition alone. Given the similarities of valproate uptake with the uptake of MCT substrates by members of the MCT family found by us and other authors at different cell types, the questions arise (i) whether valproate is an MCT substrate and if so (ii) whether an MCT is the predominant transporter involved in valproate absorption. To discuss this question, both the precise localizations of MCT at the intestinal epithelium and at the blood–brain barrier as well as their substrate specificity have to be considered. The MCT family (gene family SLC16) is composed of 14 members with each isoform possessing unique expression, biochemical properties and transport characteristics: MCT1–9, MCT11–14 and T-type amino acid transporter TAT1 [14]. So far, only MCT1–4 have been demonstrated to transport monocarboxylates in a H^+-symport mode. MCT8 is a thyroid hormone transporter instead of a monocarboxylate transporter [28,39]. For isoforms MCT5,7,9 and 11–14 the cDNA sequence but not the specific function is known; they are so-called orphan transporters (for review see [15,28]). At Caco-2 cells expression of mainly MCT1 but also MCT3–6 has been reported [16,40]. According to Gill and coworkers [16] MCT1 is expressed in the apical membrane of Caco-2 cells and the human intestine. MCT3, MCT4 and MCT5 are expressed at the basolateral membrane of the human small intestine. It should be noted, however, that several immunolocalization studies of MCT1 at the human small intestinal epithelium have provided conflicting observations, some authors favoring basolateral localization (for review see [18]). In the brain, MCT1, MCT2 and MCT4 have been described [28]. MCT1 protein is abundantly expressed in endothelial cells forming microvessels [28]. Expression of MCT2 remains controversial. MCT4 seems to be expressed exclusively in astrocytes. Therefore, the most likely MCT for uptake of monocarboxylates such as valproate from blood to brain is MCT1 provided that the system accepts valproate as a substrate. In this study, the most crucial information regarding this question is derived from the substrate specificity results (Table 1): L-lactate, pyruvate, γ-hydroxybutyrate and nicotinate inhibited valproate uptake at RBE4 cells but not at Caco-2 cells. L-Lactate and pyruvate are substrates of MCT1–4 although with different affinities (L-lactate K_m values: 3–5, 0.7, 6, 28 mM, respectively [15]). In similar studies on monocarboxylate transport in Caco-2 cells, L-lactate (10 mM) strongly inhibited H^+-stimulated [^{14}C]butyrate uptake by MCT1 [40,41]. Pyruvate is a high affinity substrate of MCT1 and MCT2 with K_m values of 0.7 or 0.1 mM, respectively, and a low affinity substrate of MCT4 (K_m = 150 mM). Because in this study neither L-lactate or D-lactate (30 mM) nor pyruvate (10 mM) affected valproate uptake in Caco-2 cells, we had to conclude that MCT1–3 are not involved in intestinal valproate absorption. So far, MCT4, a system that displays a very low affinity to L-lactate and pyruvate [15] could be responsible for valproate uptake. However, as stated above, MCT4 is not expressed in the apical membrane of Caco-2 cells. The MCT1 and MCT2 and anion exchanger inhibitor 4,4′-diisothiocyano stilbene-2,2′-disulfonic acid (DIDS) also showed no effect on valproate uptake at Caco-2 cells (Table 1). Therefore, significant participation of a DIDS-sensitive anion exchanger or MCT in intestinal valproate transport can be excluded. MCT6 can be excluded because when expressed in *Xenopus laevis* oocytes, this system transports nateglinide and probenecid, two potent inhibitors of valproate uptake, but not valproate or salicylate [42]. Furthermore, in our study, the important MCT6 substrate bumetanide had no effect on valproate uptake at Caco-2 cells. MCT8 which might be expressed at the intestine [15] and transports L-triiodothyronine with very high affinity (K_m around 1–4 μM [15,39]) can be ruled out because L-triiodothyronine (50 μM) did not interact with valproate uptake (Table 1). MCT10 mediates transport of aromatic amino acids like L-phenylalanine [15] but L-phenylalanine did not affect intestinal valproate uptake. Furthermore, whereas valproate uptake is strongly dependent on a H^+ gradient, MCT6, 8 and 10 are very likely H^+ gradient independent facilitated uniporters [15].

As stated above, Na^+-dependent carriers for monocarboxylates such as SMCT1 can be excluded because valproate uptake was completely independent of a Na^+ gradient. Considering the H^+-dependent solute symporters families in general, it might be speculated that the H^+-coupled amino acid transporter PAT1 [35] is involved in valproate transport because it has been shown that mouse PAT1 and PAT2 transport short-chain fatty acids such as acetate, propionate and butyrate in an electroneutral transport mode [43]. However, the PAT1 substrates L-proline and GABA did not affect valproate uptake in Caco-2 or RBE4 cells (Table 1). Furthermore, analyzing the substrate specificity data, organic cation transporters (OCT or OCTN, see *N*-methyl-4-phenylpyridinium, tetraethylammonium and carnitine), GABA transporters (GAT) and organic anion transporters (OAT, see *p*-aminohippurate) can be excluded. It has been suggested [18] that valproate might be a substrate for the organic anion transporting polypeptide OATP2B1. Indeed, valproate inhibits the transport of the prototype substrate estrone-3-sulfate by OATP2B1 in Caco-2 cells [17]. But, as stated above, such inhibition does not mean that valproate itself is transported by OATP2B1. To the contrary, we found – also at Caco-2 cells – that estrone-3-sulfate (10 mM) does not affect valproate uptake and conclude that the organic anion transporting polypeptide transporter is not responsible for valproate transport.

Yabuuchi and coworkers have shown that valproate is transported by anion exchanger 2 [34]. This transporter, however, is DIDS-sensitive [34]. DIDS did not affect valproate uptake in Caco-2. Secondly, anion exchanger 2 is most likely expressed at the basolateral membrane.

Valproate uptake at Caco-2 cells is similar to the mevalonate transport at rabbit intestinal brush border membrane vesicles, a compound for that Tamai and coworkers [13] observed the participation of both a proton-coupled transporter and a DIDS-insensitive anion antiporter. It could be speculated that for valproate absorption an orphan transporter of the MCT family that does not accept L-lactate as substrate and/or a DIDS-insensitive anion exchanger is responsible. We cannot conclude that MCT1 transports valproate as it has been suggested by others [18,31]. If it does, then MCT1 is not localized at the apical side of Caco-2 cells and another system is the actual valproate transporter.

The situation is very different at the blood–brain barrier. At RBE4 cells, L-lactate, pyruvate and γ-hydroxybutyrate decreased valproate uptake suggesting contribution of MCT1, 2 or 4. Since only MCT1 seems to be expressed at brain endothelial cells [28] our results are consistent with the hypothesis that MCT1 is the main valproate uptake system at the blood–brain barrier. Efflux of valproate into the interneuronal fluid might also be mediated by MCT1: this transporter has been found both in the luminal and in the abluminal membrane of brain endothelial cells [44]. It should be noted that also in the RBE4 cell line used in this study MCT1 is not only expressed very strongly but also is the predominant MCT isoform as shown by immunocytochemistry and RT-PCR studies [45].

The kinetic parameters found in this study seem reasonable. Covanis and coworkers [7] assessed serum levels of valproate in seizure-free patients. The optimum serum level was between 60 and 120 mg/l, i.e. 0.36–0.72 mM. The K_t for valproate uptake at endothelial cells measured in this study is in this range. This means that at therapeutic serum concentrations valproate uptake at the blood–brain barrier occurs at half-maximum velocity. With regard to the intestinal epithelium it is more difficult to estimate the local drug concentration in the fluid contacting the epithelial cell layer. In the study by Covanis and coworkers [7], most patients received

between 20 and 30 mg/kg. If the daily dose does not exceed 40 mg/kg or 2.5 g, it is singularly free from serious side effects [7]. Assuming a dose of 1.5 g given to a patient once a day, a luminal concentration of 0.5–2 mM is very conceivable. This concentration corresponds to the affinity constant of valproate at Caco-2 cells.

We conclude that after oral administration valproate is accumulated in intestinal cells against a concentration gradient by the activity of a specific H^+-dependent DIDS-insensitive transport system for monocarboxylates. Valproate efflux across the basolateral cell membrane probably occurs via MCT4 and/or 5. This transport mechanism is predominantly an absorptive process rather than a secretory process and explains the high oral bioavailability of valproate. The passage of valproate across the blood–brain barrier to reach therapeutic concentrations in close vicinity of target neurons seems mainly the result of the activity of MCT1 both at the luminal and at the abluminal membrane of endothelial cells.

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