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Biochemical Pharmacology 70 (2005) 1851–1860
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Drug specificity and intestinal membrane localization of human organic cation transporters (OCT)

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Received 10 June 2005; accepted 6 September 2005

Abstract

This study was performed to investigate which human organic cation transporter, hOCT1, hOCT2 or hOCT3, participates with regard to cation specificity and membrane localization in the intestinal absorption of orally available cationic drugs. Inhibition of *N*-[methyl-^{3}H]4-phenylpyridinium ([^{3}H]MPP$^+$) uptake by various compounds into Caco-2 cells and into cells (HEK-293 or CHO) that were stably transfected with hOCT1, hOCT2 or hOCT3 was compared. The uptake of [^{3}H]MPP$^+$ into Caco-2 cells was inhibited by atropine, butylscopolamine, clonidine, diphenhydramine, etilefrine, quinine and ranitidine with IC_{50} values between 6 μM and 4 mM. Transepithelial, apical to basal flux of [^{3}H]MPP$^+$ across Caco-2 cell monolayers was also strongly inhibited by these compounds. The inhibitory potency of the cationic drugs and prototypical organic cations at Caco-2 cells correlated well with the inhibitory potency measured at CHO-hOCT3 cells but much less with that at HEK-hOCT1 and -hOCT2 cells. This is functional evidence for the predominant role of hOCT3. Etilefrine and atropine were specifically transported into CHO cells by hOCT3. In Caco-2 cells, the mRNA of all three hOCT and the proteins hOCT2 and hOCT3 were detected. More importantly, immunocytochemical analyses of human jejunum revealed for the first time that hOCT3 is localized to the brush border membrane whereas hOCT1 immunolabeling was mainly observed at the lateral membranes of the enterocytes.

Keywords: Drug transport; Cationic therapeutic drugs; OCT3; Intestine; Caco-2 cells; Stably transfected cell lines

1. Introduction

The majority of drugs for therapeutic use including many antihistaminics, antacids, antiarrhythmics, antihypertensives and anticholinergics are organic cations or weak bases, i.e. molecules with a transient or permanent positive net charge. The bioavailability of cationic drugs that are weak bases such as clonidine, diphenhydramine, etilefrine, quinine, quinidine and ranitidine after oral administration ranges from 35 to 75% [1–6]. This is rather surprising considering the fact that biological membranes restrict transmembrane diffusion of organic molecules that are charged at physiological pH. It is very well known, however, that membrane transporters play an important role in drug absorption across the gastrointestinal tract. One of the best known examples is the cephalosporine and prodrug transport via the intestinal proton-coupled peptide

Abbreviations: ATRP, Atropine; BUTS, Butylscopolamine; CLON, Clonidine; DIPH, Diphenhydramine; MPP$^+$, *N*-Methyl-4-phenylpyridinium; QUID, Quinidine; QUIN, Quinine; RANT, Ranitidine; hOCT, Human organic cation transporter(s); CE, Capillary electrophoresis; HEPES, 4-(2-(Hydroxyethyl)-piperazine)-1-sulfonic acid; Tris, Tris(hydroxymethyl)aminomethanchloride

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doi:10.1016/j.bcp.2005.09.011

transporter PEPT1 [7,8]. Main candidates as membrane transporters for cationic drugs are the organic cation transporters (OCT) of the *SLC22* family OCT1, OCT2 and OCT3 also called EMT [9–11]. OCT mediate electrogenic and sodium independent translocation of organic cations in both directions across the plasma membrane. OCT1 and OCT2 have been first cloned from rat kidney [12,13]. Gorboulev et al. [14] cloned the human OCT1 and OCT2 and reported that hOCT1 was mainly expressed in human liver, whereas hOCT2 was expressed in the kidney. The organic cation transporter OCT3 was isolated from rat placenta [15]. OCT3 (or EMT) displays a broad tissue distribution: human liver, heart, brain cortex and rat intestine [16,17]. To date, the precise membrane localization of OCT3 at the intestine had not been determined.

The aim of this study was to identify the organic cation transporters that recognize cationic drugs at the human intestinal epithelial barrier. Functionally, the hOCT can be differentiated by their substrate specificity [11,18]. The intestinal cell line Caco-2 was chosen as a model for intestinal epithelial uptake and flux studies using the permanently charged prototypical organic cation MPP^+ as a model substrate [19–21]. In addition, we used transfected cell lines (HEK-293 or CHO) heterologously expressing either hOCT1, hOCT2 or hOCT3 and we demonstrate the localization of hOCT3 in the brush border membrane of human small intestinal epithelial cells.

2. Materials and methods

2.1. Materials

The cell line Caco-2 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *N*-[Methyl-^{3}H]4-phenylpyridinium acetate (specific activity 80–85 Ci/mmol) was purchased from Biotrend (Germany). Atropine, butylscopolamine, quinine and quinidine were obtained from Sigma Chemie (Deisenhofen, Germany), etilefrine and ranitidine from Synopharm (Barsbüttel, Germany) and diphenhydramine and clonidine from ICN (Eschwege, Germany). Cell culture materials were obtained from Gibco Life Technologies (Karlsruhe, Germany) and Biochrom (Berlin, Germany).

2.2. Cell culture

Cells were maintained at 37 °C in a humidified 5% CO_2, 95% air atmosphere. HEK-293 cells (American Tissue Culture Collection, Manassas, VA) containing the empty plasmid vector (pRc-CMV; Invitrogen, Groningen, The Netherlands) or stably transfected with hOCT1 or hOCT2 were established as described [22–24]. hOCT3 was kindly provided by Ganapathy and coworkers [25]. hOCT3 was subcloned into pcDNA3.1(+) (Invitrogen, Groningen, The Netherlands) and stably expressed in chinese hamster ovary (CHO) cells obtained from the American Tissue Culture Collection (Manassas, VA, USA). For Caco-2 cells the growth medium was minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution and 45 μg/ml gentamicin [26,27]. CHO cells were cultured in Iscove's modified Dulbecco's medium, supplemented with 10% fetal bovine serum, 0.6 mg/ml geneticin, 1% penicillin/streptomycin solution and 2 mM glutamine, HEK cells in Dulbeccos modified medium (low glucose) supplemented with 10% fetal bovine serum, 0.6 mg/ml geneticin, 1% penicillin/streptomycin solution and 4 mM glutamine. For transport studies, Caco-2 cells were subcultured in 35 mm petri dishes at a seeding density of 0.8×10^6 cells/dish. Uptake was measured 6 days after confluence. Caco-2 cells were also cultured on permeable TRANSWELL® cell culture inserts (diameter 24.5 mm, pore size 3 μm, Costar GmbH, Bodenheim, Germany) with a cell density of 0.2×10^6 cells/filter for 21 days as described [8,27]. CHO cells were subcultured in 35 mm petri dishes after being released with PBS containing 0.02% EDTA. The seeding density was 0.5×10^6 cells/dish. The medium was changed every day. Monolayers were used for experiments at the 7th day.

HEK cells were subcultured in 175 cm^2 culture flasks after being released with PBS. The seeding density was 2.4×10^6 cells/flask for HEK-hOCT2 cells and 9.6×10^6 cells/flask for HEK-hOCT1 cells. The medium was changed every second day. HEK cells in suspension were used for uptake studies (cell filtration) on the 3rd or 7th day after seeding, respectively. Protein concentration was adjusted to 6–16 mg/ml in uptake buffer for filtration uptake assays.

2.3. Transport studies

Cell monolayers (Caco-2 cells and CHO cells) were rinsed one time 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 HEPES/Tris (pH 7.5). To initiate uptake, 1 ml buffer containing [^{3}H]MPP^+ (1–4 nM) and/or unlabeled drugs at increasing concentrations were added to each dish. Monolayers were incubated for the times indicated at room temperature (uptake of [^{3}H]MPP^+) or at 37 °C (uptake of non-radiolabeled compounds). The uptake was stopped by aspirating the uptake buffer and washing the dishes four times with ice-cold buffer. Uptake of [^{3}H]MPP^+ (20 nM) into HEK cells was performed at room temperature by a rapid filtration technique [28] using Millipore filters (APFA type). Uptake was initiated by mixing 80 μl of cell suspension with 320 μl uptake buffer (pH 7.5) containing the radiolabeled compound and unlabeled drugs at increasing concentrations. Uptake was terminated by adding 5 ml of ice-cold buffer and filtration of the mixture. The filter was washed two times with 5 ml of the ice-cold buffer.

Transepithelial flux of [^{3}H]MPP$^+$ across Caco-2 cell monolayers cultured on permeable filters was measured as described [8,27]. Uptake was started by adding buffer (pH 7.5) containing MPP$^+$ in labeled form and the drugs to the apical compartment. At time intervals of 10–120 min samples were taken from the receiver compartment. J_{a-b} represents the flux rates from apical to basolateral compartment. After 2 h, the filters were washed, cut out of the plastic insert and also prepared for analysis. Uptake of [^{3}H]MPP$^+$ into the cell monolayer in 2 h from the apical fluid is calculated as J_{a-c}.

2.4. Analyses

In case of radiolabeled MPP$^+$ uptake, cells were solubilized in 1 ml of Igepal Ca-630 (0.5% v/v in buffer) and prepared for liquid scintillation counting. In case of uptake of non-radiolabeled compounds, cell monolayers were covered with 1 ml of distilled water, frozen and thawed twice, homogenized using a syringe with a 25-gauge needle and centrifuged at 13,000 rpm for 45 min. The supernatants of the samples from non-radioactive flux experiments were diluted 1:10 with distilled water. The samples were analyzed by high performance capillary electrophoresis on a BIO-FOCUS 3000 (Bio-Rad Laboratories, USA) equipped with a high-speed scanning, multi-wavelength detector [29]. Separations were carried out using a fused silica capillary (Bio-Rad, inner diameter 50 μm, length 50 cm). A voltage of 20 kV and a detection wavelength of 200 or 225 nm (ranitidine) were applied. The protein concentration in cell monolayers was measured by the method of Bradford.

2.5. Reverse transcriptase polymerase chain reaction

Total RNA was isolated from Caco-cells (5×10^6–1×10^7 cells/onset, $n = 4$) using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After destruction of contaminating DNA (1 U DNase/μg total RNA; Gibco-BRL, Karlsruhe, Germany), RNA was transcribed by Superscript RNase H$^-$ reverse transcriptase (100 U/μg RNA, Gibco-BRL) for 50 min at 42 °C. The reverse transcriptase was inactivated by heating to 70 °C for 15 min. The cDNAs were amplified with gene-specific primer pairs covering at least one intron (MWG Biotech, Ebersberg, Germany). For subsequent PCR, 2.5 μl buffer II, 2 μl $MgCl_2$ (15 mM), 0.625 μl dNTP (10 mM), 0.625 μl of each primer (10 μM) and 0.125 μl AmpliTaq Gold polymerase 5 U/μl (all reagents from Perkin-Elmer, Langen, Germany), were supplemented with H_2O to a final volume of 25 μl. Cycling conditions were 12 min at 95 °C, 40 cycles with 45 s at 95 °C, 45 s at 55 °C (hOCT3), 57 °C (hOCT1), 59 °C (GAPDH) and 63 °C (hOCT2), 45 s at 72 °C, and a final extension at 72 °C for 7 min. No products occurred in control reactions without template and if the reverse transcriptase was omitted. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a positive control for efficiency of RNA isolation and cDNA synthesis. The following primer pairs were used: (1) hOCT1-forward 1684 (5′-GACGCCGAGAACCTTGGG-3′) and hOCT1-reverse 1845 (5′-GGGTAGGCAAGTATGAGG-3′, accession number NM_003057), 2) hOCT2-forward 689 (5′-CTACAGTCCTCATAAATGCTGCAGC3′) and hOCT2-reverse 1102 (5′-GCTGAAGGGAGGCGGGTAGAG-3′, accession number NM_153191), 3) hOCT3-forward 1432 (5′-GGAGTTTCGCTCTGTTCAGG-3′) and hOCT3-reverse 1647 (5′-GGAATGTGGACTGCCAAGTT-3′, accession number: NM_021977), 4) GAPDH-forward 1143 (5′-CGTCTTCACCACCATGGAGA-3′) and GAPDH-reverse 1442 (5′-CGGCCATCACGCCACAGCTT-3′, accession number: AF199235). The PCR products were separated by electrophoresis on a 1.2% TRIS–acetate–EDTA gel.

2.6. Antibodies

For immunolocalization of hOCT1 we used an affinity-purified antibody against rat OCT1 from Alpha Diagnostic (San Antonio, TX, USA) that cross-reacts with hOCT1expressed in HEK-293 cells [30] but does not react with overexpressed hOCT2 or hOCT3 [30]. The antibodies against hOCT2 were raised against amino acids 533-547 (CQRPRKNKEKMIYLQV) of hOCT2 [14] and against a 21-amino acid sequence in the large intracellular loop of rOCT2 (Alpha Diagnostic). In Caco-2 cells and human small intestinal epithelium, identical results were obtained with the antibodies against rOCT2 from Alpha Diagnostic and our own previously described antibody against hOCT2. Both react with hOCT2 overexpressed in HEK-293 cells but do not react with overexpressed hOCT1 or hOCT3 [30]. The antibody against hOCT3 was raised against amino acids 297-313 (TRKKGDKALQILRRIAK) of hOCT3 [16,30]. Affinity purification of the antibodies and the demonstration that each antibody reacts specifically with the respective human OCT-subtype under the experimental conditions used for immunohistochemical detection is described elsewhere [30]. A monoclonal (mouse) antibody against villin (clone ID2C3) was obtained from Immunotech (Marseille, France). The following secondary antisera were used: Cy3-conjugated donkey–anti-rabbit IgG (Chemicon, Temecula, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey–anti-mouse IgG (Dianova, Hamburg, Germany).

2.7. Immunofluorescence

Caco-2 cells grown on plastic dishes for 6 days after confluence were harvested, pelleted by mild centrifugation in 1.5 ml reaction cups, and shock-frozen in liquid nitrogen. From a segment of human jejunum that was surgically removed during preparation of a small gastrointestinal

anastomosis, a part was taken with consent of the patient. The tissue was shock-frozen in isopentane that had been cooled in liquid nitrogen. Cryosections from the cell pellets and small intestinal tissue were prepared at 10 μm thickness, dipped in acetone at −20 °C and incubated for 1 h in 50% normal porcine serum in 0.05 M phosphate-buffered saline (PBS). Primary antibodies were diluted in PBS (affinity-purified anti-rOCT1 1:20, affinity-purified anti-hOCT2 1:400, affinity-purified anti-rOCT2, 1:400, anti-hOCT3 1:500, monoclonal mouse-anti Villin clone ID2C3 1:100) and applied at room temperature for 12–16 h. The antibodies were applied either alone or in combination for double labeling immunofluorescence. Secondary antisera were applied for 1 h at room temperature. Sections were rinsed, cover slipped with carbonate-buffered glycerol (pH 8.6), and evaluated by epifluorescence microscopy or with a confocal laser scanning microscope (TCSSP2 AOBS, Leica, Mannheim, Germany). Negative controls were done by (a) omitting the first antibodies and (b) preabsorption of the primary antibodies (anti-rOCT1, -rOCT2, -hOCT3) with 10–200 μg/ml of their corresponding peptide.

2.8. Data analysis

Data shown are means ± S.E.M. determined from at least three measurements. IC_{50} values (i.e. the concentration of the unlabeled compound necessary to inhibit 50% of specific, carrier mediated [^{3}H]MPP$^+$ uptake) were determined by non-linear regression. Transepithelial [^{3}H]MPP$^+$ flux in TRANSWELL® chambers was calculated after correction for the amount taken out from the receiver well. Statistical analysis of differences was done by the two-tailed nonparametric *U*-test. A $p \leq 0.05$ was considered significant.

3. Results

3.1. Inhibition of [^{3}H]MPP$^+$ uptake into Caco-2 cells by cationic drugs

The mRNAs of the three OCT subtypes have been detected in intestinal Caco-2 cells ([18,20,21], this study). We investigated whether the three subtypes interact equally with organic cations. First, we measured the concentration dependence for the inhibition of [^{3}H]MPP$^+$ uptake by various cations and compared it with the cloned transporters (see below). Under the experimental conditions used in our experiments, uptake of [^{3}H]MPP$^+$ into Caco-2 cells grown on plates was linear for more than 10 min (data not shown). In substrate saturation experiments using [^{3}H]MPP$^+$ (4 nM) and unlabeled MPP$^+$ (0–10 mM) an affinity constant for MPP$^+$ uptake (K_t) of 21 ± 3 μM was obtained. This value corresponds very well with the Michaelis-Menten constant of MPP$^+$ uptake at the cloned hOCT3 in transfected cells determined by Wu et al. [25] and many others [11] but not with the K_m value published by Martel et al. [19]. The activity of the individual subtypes in Caco-2 cells can be roughly estimated from inhibition of [^{3}H]MPP$^+$ uptake by inhibitors with different affinities for the subtypes. MPP$^+$ is transported by the three human subtypes with similar K_t values [11]. We measured the inhibition of [^{3}H]MPP$^+$ uptake by different concentrations of atropine, butylscopolamine, diphenhydramine, clonidine, etilefrine, quinine and ranitidine using an incubation time of 10 min (pH 7.5). IC_{50} values were obtained that ranged from 6 μM for the previously characterized hOCT inhibitor quinine [11,31] to 4 mM for the antihypotensive drug etilefrine (Fig. 1, Table 1).

3.2. Inhibition of apical to basal flux of [^{3}H]MPP$^+$ across Caco-2 monolayers by cationic drugs in the apical compartment

We tried to mimic effects of cationic drugs within the intestinal lumen on cation absorption using Caco-2 cell monolayers. Inhibition of apical to basal transepithelial [^{3}H]MPP$^+$ flux by cationic drugs (applied to the apical cell side) as well as inhibition of MPP$^+$ uptake into Caco-2 cells (across the luminal membrane) by apical drugs was measured (Fig. 2). For these experiments Caco-2 cells were cultured for 21 days on permeable filters. At this stage the transepithelial electrical resistance of the Caco-2 cell monolayers was 457 ± 16 Ω × cm^2. Net transepithelial flux of [^{3}H]MPP$^+$ (2 nM) in apical to basolateral direction (J_{a-b}) and uptake of [^{3}H]MPP$^+$ into the cells (J_{a-b}) was measured in the absence or presence of cationic drugs. The transepithelial [^{3}H]MPP$^+$ flux of 2%/h/cm^2 (Fig. 2) was strongly inhibited by all tested drugs added at high concentrations to the apical compartment (inhibition of flux rate: 0.5 mM diphenhydramine 75%, 3 mM quinine 81%, 3 mM quinidine 68%, 20 mM atropine 85%, 20 mM ranitidine 80%, 20 mM clonidine 79%, 20 mM butylscopolamine 78%). For inhibition of apical [^{3}H]MPP$^+$ uptake into

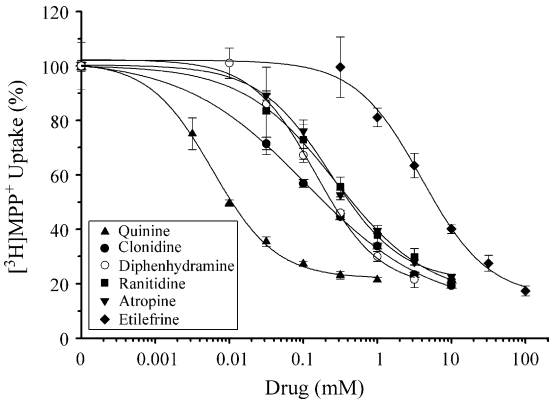

Fig. 1. Inhibition of [^{3}H]MPP$^+$ uptake into Caco-2 cells by different concentrations of various cationic drugs. Uptake of [^{3}H]MPP$^+$ (4 nM, 10 min, pH 7.5) into confluent monolayers of Caco-2 grown on plastic dishes was measured in the absence or presence of increasing concentrations of unlabeled cationic drugs. Data are means ± S.E.M. ($n = 4$).

Table 1
IC_{50} values of cationic drugs competing with the uptake of [^{3}H]MPP$^+$ in HEK cells transfected with hOCT1 or hOCT2, in CHO cells transfected with hOCT3 and in Caco-2 cells

Drug	IC_{50} (μM)				$IC50_{Caco\text{-}2}/IC50_{hOCT1}$	$IC50_{Caco\text{-}2}/IC50_{hOCT2}$	$IC50_{Caco\text{-}2}/IC50_{hOCT3}$
	hOCT1	hOCT2	hOCT3	Caco-2			
Atropine	1.2 ± 0.2	29 ± 3	466 ± 56	230 ± 28	192	7.9	0.49
Butylscopolamine	16 ± 6	764 ± 65	n.d.	n.d.	–	–	–
Diphenhydramine	3.4 ± 0.6	15 ± 3	695 ± 44	145 ± 25	42.6	9.7	0.21
Clonidine	6.5 ± 1.3	23 ± 5	110 ± 13	109 ± 14	16.8	4.7	1.0
Etilefrine	447 ± 57	4009 ± 541	4448 ± 141	3956 ± 817	8.9	0.99	0.89
Quinine	13 ± 0.8	23 ± 3	37 ± 2	5.9 ± 0.5	0.46	0.26	0.16
Ranitidine	28 ± 2	1617 ± 161	372 ± 44	271 ± 42	9.7	0.17	0.73

Uptake of [^{3}H]MPP$^+$ (HEK cells: 20 nM, CHO cells: 1 nM, Caco-2 cells: 4 nM) was measured as described in Section 2 at increasing concentrations of unlabeled cationic drugs. IC_{50} values were determined from dose-response inhibition curves as shown for Caco-2 cells in Fig. 1. Parameters are shown ± S.E. ($n = 4$).

the cells after 2 h incubation, similar degrees of inhibition (by 75–85%) were observed for all cations with the exception of 0.5 mM diphenhydramine (by 37%, Fig. 2, inset). The data suggest that uptake of MPP$^+$ across the luminal membrane of Caco-2 cells is rate limiting for transepithelial transport.

3.3. Specificity of MPP$^+$ uptake into cells stably expressing hOCT1, hOCT2 or hOCT3

We next investigated the interaction of the drugs with [^{3}H]MPP (1–20 nM) uptake by individual organic cation transporters that were expressed by stable transfection in HEK-293 cells and CHO cells. The obtained IC_{50} values are compiled in Table 1. The rank orders of affinities were hOCT1 > hOCT2 > hOCT3 for atropine, diphenhydramine, clonidine and quinine, hOCT1 > hOCT2 = hOCT3 for etilefrine, and hOCT1 > hOCT3 > hOCT2 for ranitidine. In Table 1 also the ratios between the IC_{50} values obtained for Caco-2 cells and for cells expressing either hOCT1, hOCT2 or hOCT3, respectively, are shown. For complete agreement of inhibitory potencies, these ratios should be close to one. This is approximately achieved for the ratios between the IC_{50} values for hOCT3 and Caco-2. Calculating the means ± standard deviations for the ratios obtained with different inhibitors, values of 45 ± 73 (Caco-2/hOCT1), 4 ± 4 (Caco-2/hOCT2), and 0.6 ± 0.3 (hOCT3/Caco-2) were determined. It should be noted that discrepancies exist for quinine and diphenhydramine (see Section 4). To further investigate the similarity between Caco-2 cells and cells expressing hOCT, we compared the degree of inhibition of [^{3}H]MPP$^+$ uptake by each drug (1 mM, unlabeled MPP$^+$: 0.1 mM) obtained in Caco-2 cells with the degrees of inhibition obtained with the cell lines expressing hOCT1, hOCT2 or hOCT3. There was no significant correlation between the values obtained at Caco-2 cells and those at HEK-hOCT1 ($r^2 = 0.355$) or HEK-hOCT2 ($r^2 = 0.461$). In contrast, a strong correlation ($r^2 = 0.912$) was observed between inhibition of MPP$^+$ uptake at CHO-hOCT3 and at Caco-2 cells (Fig. 3). This is strong functional evidence for the prevalence of hOCT3

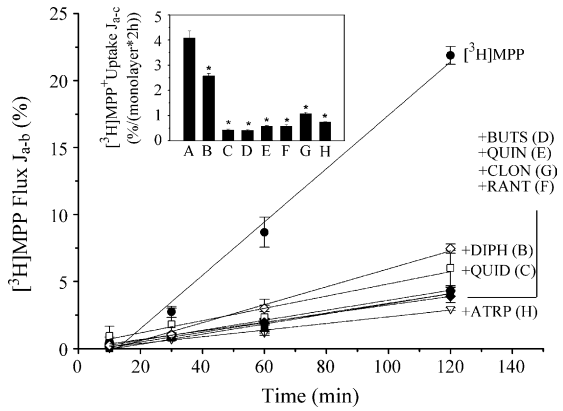

Fig. 2. Inhibition of transepithelial flux of [^{3}H]MPP$^+$ across Caco-2 monolayers by various cationic drugs. [^{3}H]MPP$^+$ (2 nM) was added with or without unlabeled cationic drugs to the apical compartment of TRANSWELL® systems (pH 7.5, 37 °C). Appearance of [^{3}H]MPP$^+$ in the basal compartment corrected for buffer replacement (transepithelial flux in apical to basolateral direction, J_{a-b}) is plotted vs. time. The insert shows uptake of [^{3}H]MPP$^+$ from the apical compartment into the cells on the filter within 2 h (J_{a-c}). (A) [^{3}H]MPP$^+$ (control); (B) plus 0.5 mM diphenhydramine; (C) plus 3 mM quinidine; (D) plus 20 mM butylscopolamine; (E) plus 20 mM quinine; (F) plus 20 mM ranitidine; (G) plus 20 mM clonidine; (H) plus 20 mM atropine. Data are means ± S.E.M. ($n \geq 3$). *Statistically different from control with $p \leq 0.05$.

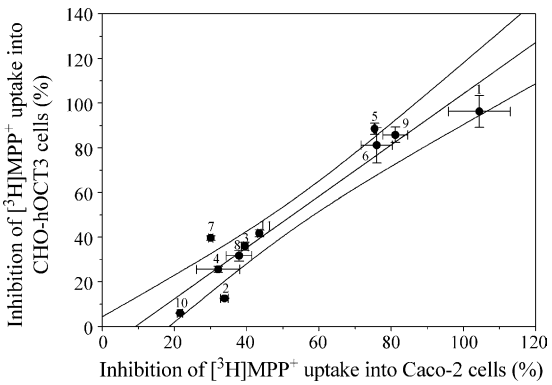

Fig. 3. Correlation between the inhibition of [^{3}H]MPP$^+$ uptake (4 nM in Caco-2, 1 nM in CHO-hOCT3 cells) by 1 mM (MPP$^+$: 0.1 mM) of various cationic drugs: (1) choline, (2) clonidine, (3) atropine, (4) quinidine, (5) butylscopolamine, (6) tetraethylammonium, (7) diphenhydramine, (8) ranitine, (9) etilefrine, (10) quinine and (11) MPP$^+$. Data are means ± S.E.M. ($n \geq 3$).

for MPP^+ uptake and for drug–hOCT3 interaction in Caco-2 cells.

3.4. Evaluation of hOCT3 activity for transport of unlabeled drugs

Having shown that the cationic drugs etilefrine, atropine, ranitidine, diphenhydramine and others interact with the three hOCT subtypes and inhibit hOCT3-mediated [^{3}H]MPP^+ uptake at Caco-2 cells, we next investigated the actual uptake of several of these drugs into cells. We first established high performance capillary electrophoresis protocols to analyze the intracellular contents. The uptake measurements were performed with CHO-hOCT3 cells in comparison with control cells that were stably transfected with the expression vector (CHO-pcDNA). The uptake of [^{3}H]MPP^+ into CHO-hOCT3 cells was at pH 7.5 with 248 ± 4 fmol/10 min per mg of protein compared to 4.3 ± 0.1 fmol/10 min per mg of protein in CHO-pcDNA cells 60-fold elevated. This indicates high expression of hOCT3 in the plasma membrane and very low background uptake of this permanently charged cation in control cells. At variance, for etilefrine, atropine, ranitidine, quinine and diphenhydramine high background uptake was observed in control CHO cells. This is probably due to high rates of simple diffusion of the drugs. After 1 h incubation at 37 °C with 0.2 mM etilefrine or 1 mM atropine significantly higher amounts of etilefrine (2-fold) and atropine (1.4-fold) were observed in CHO-hOCT3 cells compared to CHO-pcDNA cells ($P < 0.05$). In contrast, for ranitidine, diphenhydramine and quinine (all 1 mM) identical concentrations were observed. This confirms the observation that not all MPP^+ uptake inhibitors are indeed OCT substrates [9–11]. Fig. 4 shows the substrate concentration dependence of etilefrine uptake in CHO-hOCT3 cells. Uptake was measured in CHO-hOCT3 cells and CHO-pcDNA cells using a 5 min incubation period where time dependent uptake rates were linear with etilefrine concentrations between 1 and 50 mM. The amount of etilefrine taken up by CHO-pcDNA cells was subtracted from the etilefrine uptake into the CHO-hOCT3 cell line.

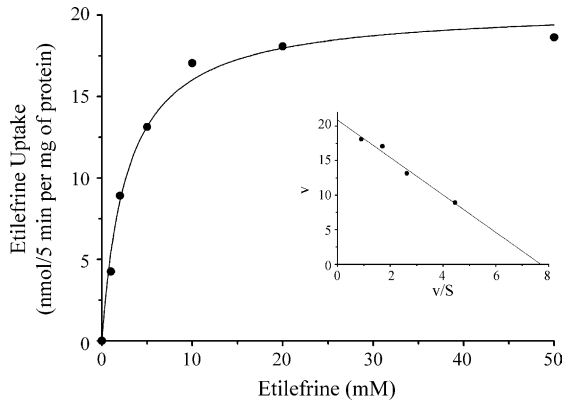

Fig. 4. Substrate saturation kinetics of etilefrine uptake by hOCT3. Uptake of etilefrine in a concentration range from 1 to 50 mM was measured at pH 7.5, 37 °C for 5 min in CHO-pcDNA cells and in CHO-hOCT3 cells. The data points were obtained by subtracting etilefrine uptake measured in CHO-pcDNA from etilefrine uptake in CHO-hOCT3 cells. Inset: Eadie–Hofstee transformation of the data: v, uptake rates in nmol/5 min/mg protein; S, etilefrine concentration in mM. The standard error of the mean values in the transfected cells ranged from 0.4 to 3.6. $n = 3$.

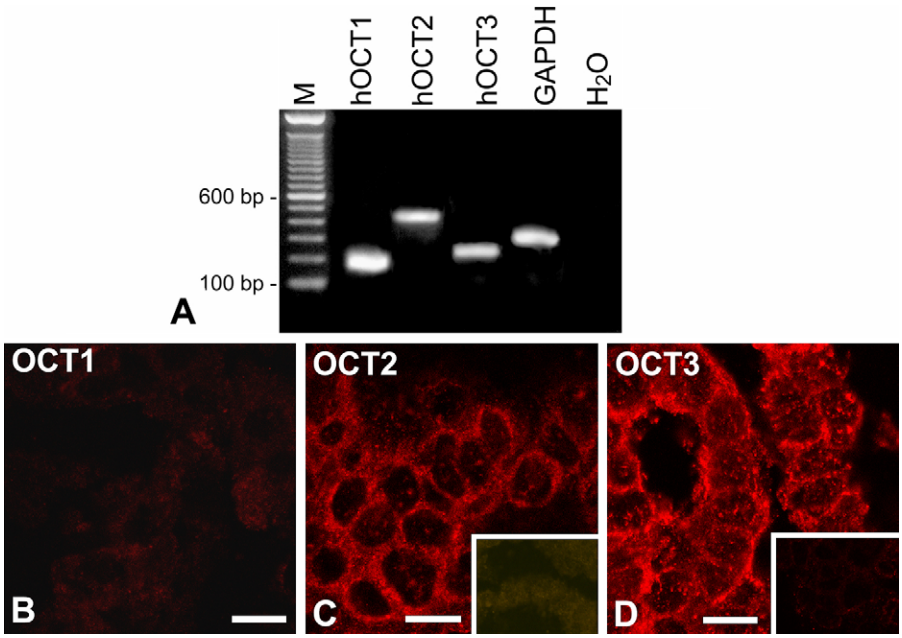

Fig. 5. Expression and localization of hOCT1, hOCT2, and hOCT3 in Caco-2 cells. (A) RT-PCR. Single bands of appropriate size were amplified by using gene-specific primers for the hOCT subtypes in Caco-2 cells. Glyceraldehyde-3-phosphate dehydrogenase specific primers were used as a positive control. (GAPDH; M standard marker, H_2O control run without template.) (B)–(D) Immunolabeling of hOCT1, hOCT2 and hOCT3 in Caco-2 cells. Insets: Antibodies preabsorbed with their respective antigenic peptides. Bars: 10 μm.

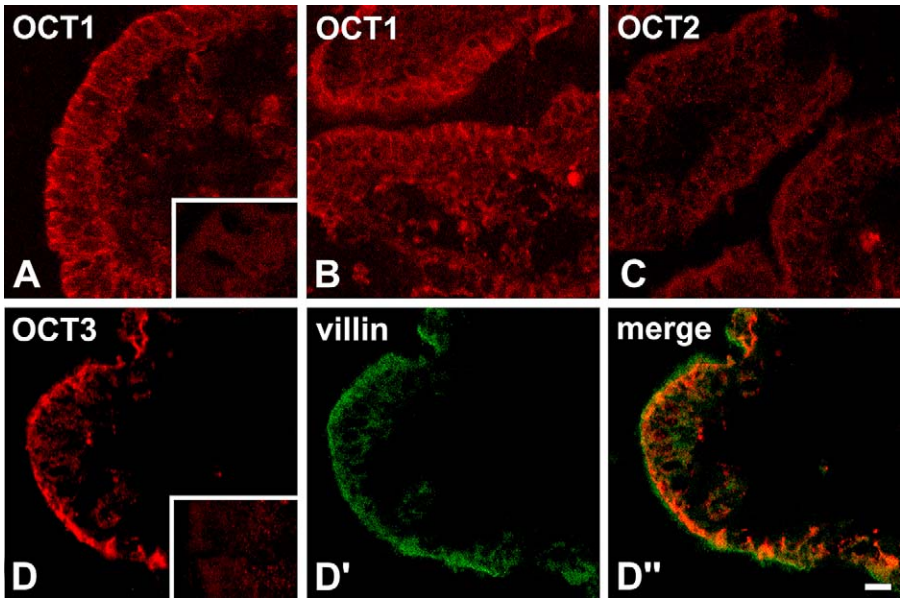

Fig. 6. Immunolabeling of hOCT1, hOCT2 and hOCT3 in human small intestine. Sections of human jejunum were stained with antibodies against rOCT1 (A, B), hOCT2 (C), and hOCT3 (D). D′ shows immunolabeling for villin and D″ double labeling for hOCT3 and villin. Insets, reactions with hOCT1 (A) or hOCT3 (D) were blocked with the respective antigenic peptides. Scale bar in D″ 10 μm for all panels.

Non-linear regression of hOCT3-mediated uptake data revealed an apparent Michaelis-Menten constant (K_t) of 2.8 ± 0.4 mM and a maximal velocity of transport (V_{max}) of 20 ± 0.7 nmol/5 min/mg of protein. For the Eadie–Hofstee plot (inset), a straight line was obtained ($r^2 = 0.99$). The K_t value of etilefrine uptake in CHO-hOCT3 cells corresponds very well with its K_i value for MPP^+ uptake inhibition in Caco-2 cells.

3.5. Expression and subcellular localization of human OCT in Caco-2 cells and human small intestinal epithelium

In differentiated Caco-2 cells mRNAs of hOCT1, hOCT2 and hOCT3 were detected by RT-PCR ($n = 4$; Fig. 5A). The length of amplified PCR products corresponded to the cDNA fragments covered by the employed primers (hOCT1 198 bp: 1648–1845, hOCT2 414 bp: 689–1102, hOCT3 216 bp: 1432–1647).

To determine protein expression and intracellular localization of transporter proteins in Caco-2 cells and human jejunum we performed immunocytochemical studies using affinity-purified antibodies that were specific for the individual hOCT subtypes. The generation and characterization of these antibodies has been described elsewhere [30]. In Caco-2 cells only very weak labeling could be observed with our antibody against rOCT1 (Fig. 5B). This indicates a much lower expression of hOCT1 protein in Caco-2 cells compared to HEK-293 cells that overexpress hOCT1 (for immunoreactivity of HEK-293 expressing hOCT1 see Fig. 3b in [30]). In Caco-2 cells, weak but significant labeling was observed with affinity-purified antibody against hOCT2 (Fig. 5C), whereas strong labeling was obtained with affinity-purified antibody against hOCT3 (Fig. 5D). Immunoreactivity for hOCT2 and hOCT3 in Caco-2 cells was detected within the cytoplasm and at the cell borders. The localization at the cell membranes suggests that hOCT2 and hOCT3 are present within the plasma membrane and/or in vesicular compartments below the plasma membrane. The immunoreactions for hOCT2 and hOCT3 in Caco-2 cells could be blocked by the respective antigenic peptides.

Caco-2 cells are of tumorous origin. Certain proteins might be over or under expressed in such a cell line compared to normal tissue. Therefore and because localization in cell pellets is inferior compared to slices of intact intestinal tissue, we then performed immunoreactions with cryosections of a normal human jejunum (Fig. 6). OCT1 immunolabeling was observed mainly in the cytoplasm and the lateral membrane of the enterocytes (Fig. 6A and B). The immunoreaction with rOCT1 could be blocked with antigenic peptide (inset in Fig. 6A). At variance to Caco-2, in human small intestinal enterocytes no specific imunolabeling was observed with affinity-purified antibody against hOCT2 (Fig. 6C). Strong OCT3 immunolabeling was found at the apical cell border of the enterocytes (Fig. 6D, D″). Double-immunolabeling with antibodies against OCT3 and villin showed that hOCT3 is located in the brush border membrane (Fig. 6D, D′ and D″). Interestingly the double labeling revealed that the very tips of the microvilli seem free of hOCT3 (Fig. 6 D″).

4. Discussion

In this investigation organic cation transporters of the *SLC22* family were identified that are capable to mediate

absorption of organic cations in the human small intestine. It has been demonstrated that hOCT3 is expressed in the apical membrane of enterocytes. The luminal localization of hOCT3 suggests that this transporter is responsible for the first step of cation absorption and the last step of xenobiotic cation excretion, respectively. Noteworthy, in differentiated bronchial epithelial cells of the lung hOCT3 is also located in the luminal membrane [30]. The strong lateral expression of hOCT1 is consistent with the previously described basolateral localization of OCT1 in enterocytes of mouse small intestine [32]. Because OCT1 and OCT2 are able to translocate organic cations in both directions across the plasma membrane and mediate cellular release of organic cations provided the ratio between the concentrations of cationic substrate in the cytosol and the extracellular space is high enough to overcome the membrane potential [22,23,30,33,34], OCT1 is most likely responsible for the release of intracellular cations into the interstitium of the mucosa. The localization of hOCT1 in the apical part of the lateral membranes may enable an accelerated release of absorbed organic cations. This may help to avoid too long contact of xenobiotics and drugs with cytosolic components of the enterocytes. However, the immunohistochemical data show that the expression of organic cation transporter subtypes might be different in Caco-2 cells compared to small intestinal enterocytes, at least in Caco-2 cells grown on plates. In Caco-2 cells no significant expression of hOCT1 protein could be observed whereas hOCT2 protein was observed at the cell borders. At variance in small intestinal enterocytes the expression of hOCT1 protein appeared to be higher than the expression of hOCT2 protein. hOCT2 might be a case where a protein is overexpressed in a cell line compared to normal small intestine.

The hypothesis about the prominent contribution of hOCT3 at cation absorption is supported by our functional data showing that the selectivity profile for inhibition of MPP^+ uptake into Caco-2 cells by several inhibitors and/or substrates of organic cation transporters correlates well to the inhibition of MPP^+ uptake at hOCT3. It has to be noted that even though the correlation hOCT3/Caco-2 is highest, significant discrepancies exist for diphenhydramine and quinine the reasons presently being unknown. Strikingly, diphenhydramine and quinine are the most lipophilic drugs among those selected in this study. A higher rate of simple diffusion into the cell can be expected. Hence, one possible explanation might be an additional effect of these compounds at intracellular proteins, e.g. inhibition of the basolaterally localized carriers for organic cation efflux from the inside. Such an effect would also easily explain the finding that the inhibitory strength of diphenhydramine on MPP^+ uptake is seemingly smaller than its effect on the transepithelial MPP^+ flux (Fig. 2). A second, very interesting hypothesis is suggested by the work of Ciarimboli and Schlatter. The authors have shown that the regulation of OCT is different in intact cells compared to transfected cells ([35], for review see [36]). Not only does the affinity of some substrates depend heavily on the expression system, probably because carriers are differently phosphorylated or minor differences in protein folding exist. Differences between expression systems are explained by different ''basal regulation status''. More interestingly for the present study, it has been shown that protein kinase C activation altered the apparent affinities of rOCT1 for carrier substrates but not for carrier inhibitors. The authors explain this effect by the presence of binding pockets with partially overlapping interaction domains for different substrates and inhibitors [36]. Quinine is a well known OCT inhibitor and not a substrate at pH 7.4 [11] and diphenhydramine may also be an inhibitor of OCT.

In addition, in the present work we characterized the interaction of drugs such as the antihypertensive etilefrine, the antihistaminic diphenhydramine and ranitidine, the parasympatholytic atropine and others that have not yet been tested before with hOCT3. The apparent affinity of these compounds is low with IC_{50} values between 0.15 mM (diphenhydramine) and 4 mM (etilefrine). Interestingly, only for etilefrine and atropine significant uptake could be observed. At present, we cannot yet preestimate the pharmacological consequences of the drug transport described here with regard to drug–drug interaction and saturation of absorption in vivo. It is well known that these drugs are orally available. The recommended oral dose of atropine is 2–3 mg and that of etilefrine 15–60 mg per day for an adult. Thereby, a luminal concentration in the middle micromolar range can be anticipated. Comparing this with the affinity constants, a complete saturation of hOCT3 and a drug–drug competition seems unlikely.

On the other hand, other transporters and simple diffusion certainly contribute to the uptake of cationic drugs in addition to hOCT3. For example, OCTN2 is expressed in the apical membrane of Caco-2 cells. MPP^+ inhibits the OCTN2-mediated uptake of many organic cations [37] but is not transported by OCTN2 itself [38]. As another example, Mizuuchi and et al. [39] observed that the specific, pH-dependent diphenhydramine uptake in Caco-2 cells was not inhibited by typical substrates for organic cation transport systems such as tetraethylammonium, cimetidine and guanidine and assigned the diphenhydramine uptake to the activity of the pH-dependent tertiary amine transport system.

In summary, we conclude that the cationic drugs of this study interact with the organic cation transporters hOCT1, hOCT2 and hOCT3. Functional evidence suggests the predominant, not exclusive role of hOCT3 for the apical uptake of MPP^+, etilefrine, atropine and other drugs at the human intestinal epithelium. Immunocytochemically we demonstrate for the first time the apical expression of hOCT3 in the human small intestine.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft grants # Br 1317/3-1 and 3-3 to MB, Li 1051/1-1 to KL and SFB487/A4 to HK. We thank Ilka Runkel, Ingelore Hamann, Ulrike Butz-Schiller and Martin Bodenbrenner for their technical help and Dr. W. Hamelman from the Surgical Clinic of the University Würzburg for supplying us with human small intestine.

This work is part of the doctoral thesis of J.M.

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