Molecular cloning of rabbit organic cation transporter rbOCT2 and functional comparisons with rbOCT1

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Zhang, Xiaohong, Kristen K. Evans, and Stephen H. Wright. Molecular cloning of rabbit organic cation transporter rbOCT2 and functional comparisons with rbOCT1. Am J Physiol Renal Physiol 283: F124–F133, 2002.—Multiple organic cation transporters (OCTs) are present in rabbit kidney and may play different functional roles. We cloned rabbit OCT2 (rbOCT2) and compared its function with that of rabbit OCT1 (rbOCT1). In transiently transfected COS-7 cells, rbOCT1 and rbOCT2 mediated uptake of [3H]tetraethylammonium (TEA) with \( K_i \) values of 188 and 125 \( \mu M \), respectively. \( n \)-Tetraalkylammonium compounds showed similar affinities for the two homologs, with \( IC_{50} \) values for inhibition of OCT1- and OCT2-mediated \([3H]TEA\) transport, respectively, of 4,538 and 1,395 \( \mu M \) for tetramethylammonium, 88.5 and 3.9 \( \mu M \) for tetrabutylammonium, and 8.8 and 7.6 \( \mu M \) for tetrpentylammonium. However, the transporters had very different affinities for cimetidine (CIM): \( IC_{50} \) of 916 and 5.7 \( \mu M \) for rbOCT1 and rbOCT2, respectively. CIM inhibition of TEA uptake into single S2 segments of rabbit proximal tubule was used to estimate the contributions of OCT1 and OCT2 to basolateral organic cation uptake. The median \( IC_{50} \) for CIM inhibition of TEA uptake was 12.3 \( \mu M \), suggesting that OCT2 is the major contributor to basolateral organic cation transport in the S2 segment of proximal tubule in rabbit kidney.

kidney; proximal tubule; uptake; tetraethylammonium; rabbit organic cation transporter

The renal tubular transport of organic substances plays an essential role in the clearance of xenobiotics, such as drugs, numerous chemicals in our environment, and some metabolites. In general, the sequence of tubular secretion of organic cations involves basolateral uptake, accumulation into the cell, and subsequent extrusion from the cell into tubular fluid across the luminal membrane of renal epithelial cells (35). Secretion and reabsorption of organic cations have been described in renal proximal tubules but may also occur in distal tubules or collecting ducts (1, 2, 6, 24). The mechanisms mediating tubular secretion have been intensively studied in various experimental preparations from rabbit kidney, including perfused (9, 24, 38) and nonperfused (5, 10, 15, 38) tubules, apical (12, 26, 29, 44, 45) and basolateral (28, 39, 44) membrane vesicles, and isolated tissue slices (21). Results from these studies identified two distinct functional classes of organic cation transporter (OCT): one localized at the basolateral membrane that is stimulated by the inside-negative membrane potential of proximal cells and the other at the brush-border membrane that is stimulated by an inwardly directed proton gradient (35).

Expression and molecular cloning strategies have been used to identify at least five OCT members from mammalian tissues, including OCT1, OCT2, OCT3, OCTN1, and OCTN2 (8). Among these members, apparent expression levels implicate OCT1 and OCT2 as major renal organic transporters that use membrane potential as a driving force. OCT1 has been cloned in the rat (17), human (46), mouse (14), and rabbit (42). OCT2, in addition to the rat, mouse, and human (13, 30, 32), has been cloned from the pig (16); OCT2 has not been cloned from the rabbit. There are distinct species differences in the tissue distribution of OCT1 and OCT2. Rat OCT1 (\( rOCT1 \)) is expressed most abundantly in the kidney, moderately in the liver, and at very low levels in the intestine (17). In contrast, human OCT1 is expressed abundantly in the liver, is scarce in the kidney, and is not evident in the intestine (13, 46). In the rabbit, OCT1 (\( rbOCT1 \)) distribution is quantitatively similar to that in the human, with highest expression in the liver, although mRNA transcripts are detectable at significant levels in the kidney and intestine (42). In the human, rat, and mouse, OCT2 expression is largely confined to the kidney (13, 30, 32). Immunocytochemistry of cortical tissue from the rat (20, 41) and human (31) confirms that OCT1 and OCT2 expression is restricted to the basolateral membrane of proximal tubule cells. In the rat, OCT1 expression is largely restricted to the early (S1) and middle (S2) segments of the proximal tubule, with OCT2 expression restricted to the S2 and S3 segments (20). Thus, in the rat, OCT1 and OCT2 are coexpressed in (at least) the S2 segment of the proximal tubule. In the human, only OCT2 expression is evident in proximal cells (31). Importantly, neither immunolocalization nor in situ hybridization provides evidence on the functional distribution of transport activity of coexpressed trans-

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porter homologs. Functional differences in expression of OCT homologs are not understood in any species. The rabbit kidney offers one of the few working models of intact renal tubule function, so it is of considerable interest to understand the physiological roles of OCT1 and OCT2 within the proximal tubule. To this end, we have cloned rabbit OCT2 (rbOCT2) and compared functional characteristics and subsegmental distribution of rbOCT1 and rbOCT2 in the proximal tubule. Although the two homologs had similar substrate specificities, rbOCT2 generally showed a higher affinity for substrates than rbOCT1. Subsegmental localization studies by PCR suggested that only rbOCT2 was expressed in S2 segments in the rabbit kidney. On the basis of the profile of cimetidine inhibition of tetraethylammonium (TEA) transport in cells expressing OCT1 or OCT2 and in isolated single S2 segments of the proximal tubule, we concluded that OCT2 is the major contributor to basolateral organic cation transport in the S2 segment of the proximal tubule in the rabbit kidney.

METHODS

Materials. [3H]TEA (13 Ci/mmol) was acquired from American Radiolabeled Chemicals (St. Louis, MO). The fluorescent organic cation 2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl trimethylammonium (NBD-TMA) was synthesized as described elsewhere (4). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). The monkey kidney cell line COS-7 was obtained from the American Type Culture Collection (Rockville, MD). The mammalian expression vector pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA). Cell culture media and all other molecular biology reagents were purchased from Life Technologies (Gaithersburg, MD). New Zealand White rabbits were purchased from Myrtle’s Rabbitry (Thompson Station, TN).

Isolation of mRNA. Total RNA was prepared from rabbit kidney tissues or isolated rabbit kidney tubules following the method of Sambrook et al. (37). Organs were removed from anesthetized animals and extensively washed or perfused with saline buffer to remove most of the remaining blood or further dissected to prepare renal tubules. Poly(A)+ RNA was selected on oligo(dT) cellulose columns from the total RNA preparation and analyzed by agarose gel electrophoresis.

Isolation of rbOCT1 and rbOCT2. The open reading frame of rbOCT1 was amplified with primers on the basis of published sequences (42) using Pfu DNA polymerase and then subcloned into pcDNA3.1 vector. To clone rbOCT2, degenerate sense and antisense oligonucleotide primers were designed from consensus sequences of human, rat, mouse, and pig OCT2 as follows: 5'-GTGGTACGACATCTGAC-3' (sense) and 5'-GATGACGAAGAGACCCGG-3' (antisense). For first-strand synthesis, 0.5 μg of rabbit kidney poly(A)+ RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (RT) H- at 37°C for 20 min. After incubation at 70°C for 15 min, ribonuclease (RNase) H was added, and the reactions were kept again at 37°C for 20 min. The RT reaction (2 μl) was used directly for amplification. The PCR solution was assembled and heated at 94°C for 3 min before Pfu DNA polymerase was added. Subsequently, PCR was performed using the following profile: 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min for 35 cycles. The last cycle was terminated after an elongation time of 7 min. A 406-bp RT-PCR product was gel purified and sequenced. To obtain the remaining 5’ and 3’ portions on the rabbit kidney OCT2 sequence, the PCR-based 5’- and 3’-rapid amplification of cDNA ends (RACE) systems (GIBCO-BRL) were utilized. Briefly, two gene-specific primers [5’-GGAGACGACACCTGCATCTTG-3’ (sense) and 5’-GAGATTCCTGATGACGTGG-3’ (antisense)] were designed from the partial rbOCT2 sequence. The 5’- and 3’-RACE reactions were primed with an internal gene-specific primer and an adapter primer. The PCRs were performed according to the manufacturer’s protocols. The RACE products were gel purified and subcloned into the mammalian expression vector pcDNA3.1. The two overlapped RACE products were digested by BamHI/EcoRI and then ligated to form a full-length cDNA of rbOCT2. rbOCT1 and rbOCT2 sequences were confirmed in the sense and antisense strands by an Applied Biosystems model 373A sequencing unit at the University of Arizona sequencing facility.

Cell culture and transfection. COS-7 cells were grown at 37°C in a humidified atmosphere (5% CO2) in plastic culture flasks. The medium was Eagle’s modification (F12K) medium supplemented with 10% fetal calf serum. The medium was changed every day, and the culture was split every 3 days. Cells were transfected with supercoiled plasmid DNA by electroporation. Briefly, cells were transfected with 10 μg of DNA at 260 V for 1,050 ms and seeded in 12-well plates at 32,000 cells/well. Uptake studies were performed 48 h after transfection (cells were generally confluent at this time). Expression of rbOCT1 or rbOCT2 was verified by RT-PCR and by visual inspection of the accumulation of the fluorescent cationic dye NBD-TMA (4).

Transport assays. Uptake was measured at 25°C. After a preincubation period of 30 min with Waymouth’s buffer (WB; in mM: 135 NaCl, 13 HEPES-NaOH, pH 7.4, 28 d-glucose, 5 KCl, 1.2 MgCl2, 2.5 CaCl2, and 0.8 MgSO4), the cells were incubated with ~76 nM [3H]labeled substrates in WB. Incubation was stopped by rinsing the cells three times with 2 ml of ice-cold WB containing 250 μM tetrapentylammonium (TPeA). The cells were then solubilized with 0.2 N NaOH and analyzed by liquid scintillation spectrometry. Uptakes are expressed as moles per square centimeter of cellular cell surface of the confluent monolayer.

Transport in isolated tubules. New Zealand White rabbits were killed by intravenous injection of pentobarbital sodium. The kidneys were flushed via the renal artery with an ice-chilled solution containing 250 mM sucrose and 10 mM HEPES adjusted to pH 7.4 with Tris base. The kidneys were removed and sliced transversely, and the slices were placed in a dish containing ice-chilled dissection buffer (in mM: 110 NaCl, 25 NaHCO3, 5 KCl, 2 NaHPO4, 1.8 CaCl2, 1 MgSO4, 10 sodium acetate, 8.3 d-glucose, 5 l-alanine, 4 lactate, and 0.9 glycine) adjusted to pH 7.4 with HCl or NaOH and gassed continuously with 95% O2-5% CO2 to maintain the pH (osmolarity ~290 mmol/kgH2O). Dissection of tubules from a slice was performed manually at 4°C without the aid of enzymatic agents. Dissections were limited to isolation of early proximal straight tubules (S2 segments), defined as extending from the cortical surface to the corticomedullary junction (38). Uptake of [3H]TEA into tubules was started by transfer of individual tubule segments to a chamber containing 37°C uptake medium with labeled substrate and, in some cases, unlabeled cimetidine. Uptake was terminated by individual transfer of the tubule segments to 10 μl of 6 N NaOH that was dispensed into microwells of a plastic 60-well plate (Nunc, Naperville, IL). A 10-μl syringe was used to transfer the NaOH solution (and the tubule segment) to separate
plastic scintillation vials that contained 300 μl of distilled water. Each microwell was rinsed twice with 10 μl of distilled water that was added to its respective scintillation vials. The radioactivity in each vial was measured using liquid scintillation spectrometry. Three to four tubule segments were used for each experimental and each control condition.

RT-PCR analysis. RT-PCR was performed with mRNA from isolated S2 segments and rbOCT1- and rbOCT2-specific primers: for rbOCT1 a 499-bp fragment derived from 5′-ATGTGTTGTCTGCGCTA-3′ (sense) and 5′-CCACTGGAACGGAGCA-3′ (antisense) and for rbOCT2 a 406-bp fragment derived from 5′-GTGCAAGYCTCTGAGATAAG-3′ (sense) and 5′-GATGCGRCRATGTGAC-3′ (antisense). Subsequent PCR was carried out in separate reactions employing identical parameters using primers for rbOCT1 or rbOCT2 and equivalent amounts of tubule RT reaction.

Data analysis. Uptake values are presented as means ± SE. In each experiment, a minimum of three wells was used to generate each data point, and each experiment was repeated at least three times.

Amino acid sequences and pairwise sequence alignments were analyzed with default parameters with the ClustalW algorithm available on the internet from Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_server.html).

RESULTS

Molecular properties of rbOCT2. A 406-bp RT-PCR product was generated by PCR amplification using degenerate oligonucleotide primers designed from consensus sequences of human, rat, mouse, and pig OCT2. PCR-based 5′- and 3′-RACE systems were utilized to obtain the remaining 5′ and 3′ portions of the rbOCT2 sequence. The two overlapped RACE products were digested by BamHI/EcoRI and then subcloned into the mammalian expression vector pcDNA3.1 to form a full-length cDNA of rbOCT2. The nucleotide and predicted amino acid sequences of rbOCT2 are shown in Fig. 1. The full-length cDNA is 2,180 bp and contains a 42-bp 5′-untranslated region, a 1,662-bp open reading frame, and a 476-bp 3′-untranslated region. It encodes a protein of 554 amino acids with a predicted mass of 61 kDa (GenBank accession no. AF458095). Assessment of possible secondary structure (TMHMM, version 2.0) (27, 40) suggests the presence of two large hydrophilic loops and 12 membrane-spanning domains, which is similar to that of rbOCT1 and other OCT2 isoforms (8). The protein sequence contains five potential N-linked glycosylation sites (N-X-T/S) at positions 71, 96, 112, 198, and 331, with the first two sites (positions 71 and 96) conserved among OCT1, OCT2, and OCT3 (8). In addition, one potential protein kinase A phosphorylation site (position 344), two potential tyrosine kinase phosphorylation sites, and three potential protein kinase C (PKC) phosphorylation sites (positions 59, 285, and 319) were identified (Fig. 1).

BLAST searches of the protein and gene databases indicated that the rbOCT2 protein belongs to the OCT family of the Major Facilitator Superfamily (2A.1.19) (36). rbOCT2 has high sequence homology with its orthologs cloned from the human, rat, mouse, and pig (82–84% identity) and strong homology with the related proteins OCT1 (68–69% for human, rat, and mouse and 71% for rabbit) and OCT3 (48–49%). Alignment of the sequences of rbOCT1 and rbOCT2 is shown in Fig. 2.

Functional comparison of rbOCT2 with rbOCT1. Transient transfection of COS-7 cells with pcDNA3.1-containing OCT2 increased the 5-min uptake of [3H]TEA by 14-fold, and this was reduced to a level comparable to that observed in the vector-only control by addition of 2.5 mM unlabeled TEA (Fig. 3). Figure 4 shows the time courses of [3H]TEA uptake into COS-7 cells transfected with rbOCT1 (Fig. 4A) or rbOCT2 (Fig. 4B). For both processes, uptake was time dependent and reasonably linear for 2–5 min. Extrapolation of the time courses to time 0 consistently revealed positive intercepts, suggesting that accumulation of labeled TEA might include a rapid binding component. However, the rapid component of cellular TEA accumulation was completely blocked by addition of unlabeled TEA with kinetics that were indistinguishable from those of the time-dependent portion of uptake (data not shown). In addition, a rapid component of accumulation was absent in experiments with wild-type COS-7 cells and cells transfected with empty vector (data not shown), indicating that it reflected expression of transport protein. Therefore, in subsequent kinetic studies, 2- and 5-min uptakes were used as estimates of the initial rates of transport mediated by OCT1 and OCT2, respectively.

Next, we examined the concentration dependence of [3H]TEA uptake by rbOCT1 and rbOCT2. The transfectants were exposed to [3H]TEA and increasing concentrations of unlabeled TEA (0–2.5 mM). Inhibition of labeled TEA uptake produced by unlabeled TEA was used to calculate the kinetics of TEA transport according to the following relationship (23)

\[
J = \frac{J_{max}[\text{T}]}{K_t + [\text{T}]} + C
\]

where \(J\) is the rate of [3H]TEA transport from a concentration of labeled substrate equal to [\text{T}], \(J_{max}\) is the maximum rate of mediated TEA transport, \(K_t\) is the TEA concentration that results in half-maximal transport (Michaelis constant), [\text{T}] is the concentration of unlabeled TEA in the transport reaction, and \(C\) is a constant that represents the component of total TEA uptake that is not saturated (over the range of substrate concentrations tested) and presumably reflects the combined influence of diffusive flux, nonspecific binding, and/or incomplete rinsing of the cell layer. For OCT1 and OCT2, addition of unlabeled TEA resulted in a hyperbolic inhibition of labeled TEA uptake with <10% of accumulated label in the “nonsaturable” component. Figure 5 shows the kinetics of rbOCT1- and rbOCT2-mediated TEA transport in representative experiments. In three separate experiments on cells expressing rbOCT1, \(J_{max}\) was 49.7 ± 2.73 pmol·cm⁻²·min⁻¹ with a \(K_t\) of 188 ± 20 μM. For rbOCT2, \(J_{max}\) was 13.1 ± 0.16 pmol·cm⁻²·min⁻¹ with a \(K_t\) of 125 ± 22 μM.
To examine the characteristics of substrate recognition, accumulation of \[^{3}H\]TEA by rbOCT1- and rbOCT2-expressing cells was measured in the presence of increasing concentrations of several organic cations. All the test agents inhibited TEA uptake by rbOCT1 and rbOCT2 cells (Table 1), suggesting that both transporters recognize a wide variety of cationic molecules. OCT2 displayed a higher apparent affinity for every compound tested. These differences are most evident by considering the ratio of IC\(_{50}\) values (inhibitor concentrations that reduced mediated uptake of \[^{3}H\]TEA by 50%, determined using the analytic method of Groves et al. (15)) obtained for rbOCT1 relative to that obtained for rbOCT2 (Fig. 6). In some cases, the ratio was comparatively small, e.g., ratios of 1.2 and 1.5 for TPeA and TEA, respectively, indicating that the two homologs had similar affinities for these molecules. However, several compounds showed substantially higher affinities for rbOCT2 than for rbOCT1. Cimetidine showed the most marked difference in apparent affinity for the two transporters (Figs. 6 and 7), with IC\(_{50}\) values of 180 times higher for rbOCT1 than for rbOCT2 (916 vs. 5.7 nM; Table 1). The fluorescent cation NBD-TMA also displayed a much higher affinity for rbOCT2 (IC\(_{50}\) 23.6 nM) than for rbOCT1 (IC\(_{50}\) 129 nM; Table 1, Fig. 6).

Distribution of rbOCT1 and rbOCT2 in the S2 segment of the rabbit proximal tubule. The marked difference in apparent affinity of cimetidine for the two rabbit OCT homologs suggested that cimetidine could...
be used as a tool to examine the relative contribution of OCT1 and OCT2 to basolateral organic cation transport in the intact proximal tubule. Figure 8 shows the effect of increasing concentrations of cimetidine on the basolateral uptake of \[^3H\]TEA into single, nonperfused S2 segments of rabbit renal proximal tubule. Cimetidine proved to be a high-affinity inhibitor of basolateral TEA uptake. In five separate experiments with tubules from different rabbits, the average IC\(_{50}\) for cimetidine’s inhibition of TEA uptake was 19.5 ± 8.4 \(\mu\)M. In one of those experiments, the IC\(_{50}\) was particularly high (52 \(\mu\)M), and we consider the median value of 12.3 \(\mu\)M to be more representative of the inhibitory effect of cimetidine on basolateral organic cation transport (at least for TEA and cimetidine) in the S2 segment of the proximal tubule in rabbit kidney.

We also compared mRNA expression of rbOCT1 and rbOCT2 in individual S2 segments of rabbit renal proximal tubule by RT-PCR. In control experiments, the primers were shown to amplify appropriately sized products using the respective cDNAs as templates (data not shown). However, when the RT material derived from single S2 segments (single tubules from 3 rabbits) was used as the template, amplified product was only apparent using the primers for OCT2 (Fig. 9), consistent with the conclusion that OCT2 is the major contributor to basolateral organic cation transport (at least for TEA and cimetidine) in the S2 segment of the proximal tubule in rabbit kidney.

Fig. 2. Sequence alignment of rbOCT1 and rbOCT2. Amino acid sequences of rabbit OCT1 (rbOCT1; accession no. AF196774, GenBank) and rbOCT2 (accession no. AF458095, GenBank) were aligned. Amino acids that are fully conserved are highlighted. Multiple sequence alignment was made using the Network Protein Sequence Analysis program.
Fig. 3. Tetraethylammonium (TEA) uptake in COS-7 cells transfected with pcDNA3.1 (vector) or pcDNA3.1/rbOCT2 was measured at room temperature with 76 nM [3H]TEA 48 h after transfection. Unlabeled TEA (2.5 mM) was added for uptake in the inhibition experiment. Each point represents mean ± SE of substrate accumulation in 3 individual experiments with 3 wells of cells for each experiment.

Fig. 4. Time course of rbOCT1- and rbOCT2-mediated transport of [3H]TEA in COS-7 cells. Uptake of [3H]TEA was measured 48 h after transfection. pcDNA3.1-rbOCT1 (A) and pcDNA3.1-rbOCT2 (B) cells were incubated for specified time periods at room temperature in buffer containing 76 nM [3H]TEA. After incubation, radioactivity [disintegrations per minute (DPM)] of solubilized cells was counted. Each point represents mean ± SE of 3 individual experiments with 3 wells of cells for each experiment.

Fig. 5. Comparison of kinetics of TEA transport in transient transfected COS-7 cells expressing rbOCT1 (A) or rbOCT2 (B). Uptakes of [3H]TEA at 2 and 5 min were measured for rbOCT1 and rbOCT2, respectively. Transport buffer contained increasing concentrations of unlabeled TEA (0–2.5 mM). For representative experiments shown, the Michaelis constant ($K_m$) for TEA transport mediated by rbOCT1 was 189 μM, with a maximum velocity ($J_{max}$) of 47.9 pmol·cm⁻²·min⁻¹. Inset: total velocity ($J_{total}$)-total TEA concentration plot of these data using a nonlinear fit to a model, consisting of one saturable (Michaelis-Menten) term plus one nonsaturable term, the analysis of which resulted in $K_m$ of 168 μM with $J_{max}$ of 44.3 pmol·cm⁻²·min⁻¹. For rbOCT2, $K_m$ was 106 μM with $J_{max}$ of 12.8 pmol·cm⁻²·min⁻¹ (inset: $K_m$ = 122 μM with $J_{max}$ = 13.1 pmol·cm⁻²·min⁻¹). Data points are means ± SE of uptakes measured in 3 wells from single representative transfections.

Table 1. Inhibition constants for organic cations to compete with [3H]TEA uptake mediated by rbOCT1- and rbOCT2-transfected COS-7 cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>rbOCT1 (μM)</th>
<th>rbOCT2 (μM)</th>
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<tbody>
<tr>
<td>Cimetidine</td>
<td>916 ± 383</td>
<td>5.7 ± 1.4</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>205 ± 25</td>
<td>62 ± 21</td>
</tr>
<tr>
<td>NBD-TMA</td>
<td>129 ± 66</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>MPP</td>
<td>39 ± 10</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>TMA</td>
<td>4,538 ± 606</td>
<td>1,395 ± 188</td>
</tr>
<tr>
<td>TEA</td>
<td>188 ± 20</td>
<td>125 ± 22</td>
</tr>
<tr>
<td>TPrA</td>
<td>88 ± 19</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>TBA</td>
<td>13.9 ± 1.4</td>
<td>5.3 ± 1.7</td>
</tr>
<tr>
<td>TPeA</td>
<td>8.8 ± 1.9</td>
<td>7.6 ± 0.7</td>
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Values are means ± SE. rbOCT1 and rbOCT2, rabbit organic cation transporters 1 and 2, TEA, tetraethylammonium; NBD-TMA, 2-(4-nitro-2,1,3-benzoxadiazol-7-yl) aminoethyl trimethylammonium; MPP, 1-methyl-4-phenylpyridinium; TMA, tetramethylammonium; TPrA, tetr-propylammonium; TBA, tetrabutylammonium; TPeA, tetr-pentylammonium.
DISCUSSION

In the kidney, organic cation transport systems play physiological and pharmacological roles in the excretion and/or reabsorption of a wide array of endogenous organic cations, cationic drugs, and cationic toxins.

Five members of the OCT family (2.A.1.19) of the Major Facilitator Superfamily (36) have been cloned in several mammalian species including human, mouse, rat, rabbit, and pig (8). However, until the present study, OCT1 was the only OCT cloned from the rabbit. Much of our understanding of the mechanisms of renal organic cation transport is based on studies performed in rabbit kidney, owing to its suitability for the study of the integrated function of the physiologically intact, isolated perfused tubule. There are now substantial data suggesting that net renal organic cation transport could reflect the concerted behavior of separate transport processes (22). However, substantial species differences in the quantitative characteristics of organic cation transport, particularly with respect to the interaction of bulkier organic cations with orthologous transporters in rodents, rabbits, and humans (11), make it imprudent to use results obtained with cloned transporters of one species to predict the transport behavior of intact tubules in another. Consequently, we cloned the rabbit ortholog of OCT2 and compared its characteristics with those of its related renal transport homolog OCT1 to provide new insights into our understanding of renal organic cation transport.

Fig. 8. Effects of cimetidine on TEA uptake by R2 segment of rabbit renal proximal tubule. Nonperfused R2 segment was incubated for 5 min at 37°C with 1.8 µM [3H]TEA in the presence of 0–1 mM cimetidine. After incubation, radioactivity of solubilized tissue was counted. Each data point is mean uptake measured in 3–4 tubule segments determined in 5 separate animals.

Fig. 9. Detection of RB OCT1 and RB OCT2 mRNA levels by RT-PCR in single S2 segments of rabbit renal proximal tubule. mRNA prepared from the tubule segment was used for 1st-strand cDNA synthesis. Subsequent PCR amplification was performed with RB OCT1- or RB OCT2-specific primers using the indicated amount of RT reaction. No RT reaction was added to negative control. PCR products for negative control, RB OCT1, and RB OCT2 were loaded on the same gel and visualized with ethidium bromide. Lanes 1 and 11, 1-kb DNA ladder; lanes 2, 4, 6, 8, and 10, RB OCT1; lanes 3, 5, 7, and 9, RB OCT2.
understanding of the molecular basis of organic cation transport in intact renal tubules.

The amino acid identity between rbOCT1 and rbOCT2 is 71%. Computer analysis reveals 2 large hydrophilic loops and 12 transmembrane-spanning α-helices in rbOCT2, which is similar to rbOCT1 (and all other members of the OCT family) (8). There are five potential N-linked glycosylation sites (N-X-T/S) at positions 71, 96, 112, 198, and 331, with the first two sites (positions 71 and 96) conserved among OCT1, OCT2, and OCT3. rbOCT2 also possesses three potential PKC phosphorylation sites at positions 59, 285, and 319, which are located in the two large hydrophilic loops. Transport studies with isolated rabbit renal proximal tubules (18) and with the cell lines IHKE-1 and LLC-PK1 (19) revealed a modulation of organic cation transport by phorbol esters, and transport activity of rOCT1 (expressed in HEK-293 cells) is increased after activation of PKC (25). On the other hand, accumulation of the fluorescent cationic dye 4-(dimethylaminostyryl)-N-methylpyridinium is decreased after activation of PKC in isolated human proximal tubules (34). However, it is not known whether rbOCT2 is regulated by PKC and which of the potential phosphorylation sites may be involved in regulation of organic cation transport by PKC.

To compare the functional characteristics of rbOCT1 and rbOCT2, we transfected rbOCT1 and rbOCT2 into COS-7 cells. The two transporters had similar apparent affinities for transport of TEA (188 vs. 125 μM, respectively; Fig. 5), and the ratio of IC_{50} values (OCT1/OCT2) was comparatively similar (i.e., ~3-fold difference or less) for several other organic cations, including the n-tetraalkylammonium compounds tetrapentylammonium, tetrabutylammonium, and tetramethylammonium (Fig. 6). However, substantial differences were noted in the relative affinity of several organic cations for OCT1 vs. OCT2. The greatest differences were noted for cimetidine, with an OCT1 IC_{50} of >900 μM compared with OCT2 IC_{50} of 6 μM (Fig. 6, Table 1), and NBD-TMA, with an OCT1 IC_{50} of 129 vs. 4 μM for OCT2 (Fig. 6, Table 1). These results confirm that in the rabbit, as in other species, OCTs display a broad specificity for cationic substrates. The results also indicate that, despite their broad specificity, steric features of selected substrates must strongly influence binding to the transport receptors of these closely related homologs.

It is interesting to compare the results obtained here for rbOCT1 and rbOCT2 with those recently reported for the rat orthologs. It should be emphasized that such comparisons need to be made cautiously. Here, we limit the comparisons to those studies that directly compared the relative affinity of rOCT1 and rOCT2 for a common set of substrates. As shown in Fig. 10, the similar affinity of rbOCT1 and rbOCT2 for TEA is a characteristic shared by the rat orthologs of these processes. Nevertheless, it is worth noting that the absolute values for K_{m}/inhibition constant/IC_{50} values for interaction of TEA with the rat OCTs varied between studies, ranging from ~100 μM (3) to 150 μM (33) when expressed in oocytes to ~50 μM when expressed in Madin-Darby canine kidney (MDCK) cells (43). The relative interaction of OCT1 and OCT2 with cimetidine produced the most striking disparities. As noted previously, in the present study with rbOCTs, there was a 160-fold difference in the IC_{50} for cimetidine inhibition of OCT1 (~900 μM) vs. OCT2 (~6 μM). In two studies comparing the interaction of cimetidine with rat OCT1 and OCT2, there was virtually no difference in the relative interaction of OCT1 and OCT2 with this compound. There was, however, a very large difference in the absolute affinity of the transporters for cimetidine, with IC_{50} of ~350 μM when they were expressed in oocytes (33) compared with IC_{50} of 6–9 μM when they were expressed in cultured MDCK cells (43). Similar degrees of variability between rabbit and rat orthologs and between different systems expressing the rat homologs were noted for interactions with 1-methyl-4-phenylpyridinium and guanidine. The extent to which these differences in relative and absolute interactions of organic cations with OCTs reflect differences in species, expression systems, and/or technique is not clear. Arndt et al. (3) recently determined the relative interaction of a wide array of substrates for rOCT1 and rOCT2 (expressed in oocytes) and noted that several compounds discriminated effectively between these two transporters (notably mepiperphenidol and O-methylisoprenaline as OCT2-selective inhibitors and corticosterone as an OCT1-selective inhibitor). They noted that such marked differences could be used to dissect out the individual contributions of these processes in intact proximal tubule preparations. However, the variability in interaction of substrates with OCTs noted above, which may reflect species and/or expression system influences, underscores the importance of using caution when results obtained with cloned transporters are used to make predictions about the experimental.

Fig. 10. Comparison of relative inhibitory interactions of TEA, cimetidine (CIM), MPP, and guanidine (Guan) with rabbit or rat orthologs of OCT1 (numerator) and OCT2 (denominator). Also compared are these interactions for the 2 rat homologs in 2 separate studies (3, 33) (cross-hatched and shaded bars, respectively) employing the oocyte expression system and a third study in which these transporters were stably expressed in cultured Madin-Darby canine kidney (MDCK) cells (43). Pairs of numbers in parentheses above each column are measured K_{i}/IC_{50} values (in μM) for OCT1 and OCT2, respectively.
expected behavior of these processes in native tissues from other species (e.g., the human).

Having emphasized this important caveat, it was nevertheless tempting to use the large difference in apparent affinity for cimetidine of rbOCT1 and rbOCT2 to examine the level of functional expression of these two homologs in the S2 segment of rabbit proximal tubule. The comparatively high affinity of the S2 segment for cimetidine (IC$_{50}$ ~ 12 μM; Fig. 8) compared closely with the high affinity of rbOCT2 (IC$_{50}$ ~ 6 μM; Fig. 7) and contrasted markedly with the low affinity of rbOCT1 (IC$_{50}$ ~ 1 mM; Fig. 7) for this compound. Again, it is important to acknowledge the underlying assumption that the OCT affinities expressed in COS-7 cells are those occurring when these processes are expressed in their native cell type. However, the similarity between the $K_t$ values for TEA transport observed for OCT1 and OCT2 in COS-7 cells (180 and 120 μM, respectively) and the $K_t$ for TEA transport in single proximal tubule segment from rabbit kidney (108 μM) (15) supports tentative conclusions based on such comparisons. Moreover, in the present case, RT-PCR showed clear expression of mRNA for OCT2 in single isolated S2 segments of rabbit proximal tubules but failed to amplify mRNA for OCT1. Taken together, these data suggest that OCT function in the S2 segment of proximal tubule is dominated by OCT2 in the rabbit.

The distribution of OCTs in the rat and human proximal tubule has also been examined. In the rat, immunocytochemistry (20, 41) and in situ hybridization (20) indicate that the S2 region of the proximal tubule contains OCT1 and OCT2. The human proximal tubule, in contrast, appears to be dominated by basolateral expression of OCT2 along the entire length of the proximal tubule (38). Although the failure to find evidence of functional expression of OCT1 in rabbit S2 segments is consistent with the expression profile of OCTs in the human tubule, OCT1 is certainly expressed in rabbit kidney. Northern blots of whole rabbit kidney (108 μM) (15) supports tentative conclusions based on such comparisons. Moreover, in the present case, RT-PCR showed clear expression of mRNA for OCT2 in single isolated S2 segments of rabbit proximal tubules but failed to amplify mRNA for OCT1. Taken together, these data suggest that OCT function in the S2 segment of proximal tubule is dominated by OCT2 in the rabbit.

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