Creatinine Transport by Basolateral Organic Cation Transporter hOCT2 in the Human Kidney

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Received January 17, 2004; accepted March 15, 2004

Purpose. Creatinine is excreted into urine by tubular secretion in addition to glomerular filtration. The purpose of this study was to clarify molecular mechanisms underlying the tubular secretion of creatinine in the human kidney.

Methods. Transport of [^{14}C]creatinine by human organic ion transporters (SLC22A) was assessed by HEK293 cells expressing hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3.

Results. Among the organic ion transporters examined, only hOCT2 stimulated creatinine uptake when expressed in HEK293 cells. Creatinine uptake by hOCT2 was dependent on the membrane potential. The Michaelis constant (K_m) for creatinine transport by hOCT2 was 4.0 mM, suggesting low affinity. Various cationic drugs including cimetidine and trimethoprim, but not anionic drugs, markedly inhibited creatinine uptake by hOCT2.

Conclusion. These results suggest that hOCT2, but not hOCT1, is responsible for the basolateral membrane transport of creatinine in the human kidney.

KEY WORDS: creatinine; glomerular filtration rate; hOCT2; organic cation transporter; tubular secretion.

INTRODUCTION

In the proximal tubules of mammalian kidney, organic ion transporters limit or prevent the toxicity of organic anions and cations by actively secreting these substances from the circulation into the urine (1–5). We isolated a second member of the organic cation transporter (OCT) family, rat (r) OCT2 (6), showing 67% amino acid identity to rOCT1 (7). Functional studies using *Xenopus* oocytes (6–10) and transfected mammalian cells (11–13) as expression systems suggested that rOCT1 and rOCT2 transport various structurally unrelated cations in a voltage-dependent fashion. rOCT1 and rOCT2 possess similar but not identical specificities for various cationic compounds. Both rOCT1 and rOCT2 protein were localized in the basolateral membrane of renal tubular cells (14,15), although the distributions of these transporters along the nephron were distinct (13).

To date, three distinct genes encoding human organic cation transporters have been identified including hOCT1, hOCT2, and hOCT3 (5). In addition, we identified hOCT2-A, an alternatively spliced variant of hOCT2, expressed in the human kidney, with different transport characteristics from that of hOCT2 (16). We also demonstrated that the mRNA level of hOCT2 was the highest in the human kidney among organic cation transporters examined, suggesting hOCT2 to be the dominant organic cation transporter in the human kidney (17). In contrast, hOCT1 is mainly transcribed in the liver, suggesting that hOCT1 is responsible for the hepatic uptake of organic cations (18–19). Although characterization of organic cation transport by hOCT2 have been done, intrinsic roles of hOCT2 in the disposition of physiological substances have not been clarified.

It is established that creatinine, a catabolic product of creatine, is eliminated predominantly into urine. Creatinine can also be secreted via the renal tubules in addition to the glomerular filtration, however, the molecules mediating tubular secretion of creatinine in the human kidney have not been identified. Because organic ion transporters recognize a wide variety of ionic compounds, thereby mediate tubular secretion of organic ions, we measured creatinine transport by organic ion transporters (SLC22A), hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3, to assess the involvement of these transporters in the tubular secretion of creatinine.

MATERIALS AND METHODS

Cell Culture

HEK293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2/95% air at 37°C. For uptake experiments, the cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cell monolayers were used at day 3 of culture for uptake experiments. In this study, HEK293 cells between the 68th and 89th passages were used.

Transfection

pCMV6-XL4 plasmid vector (OriGene Technologies, Rockville, MD, USA) DNA containing hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3 cDNA, and pBK-CMV vector (Stratagene, La Jolla, CA, USA) were purified using Marligen High Purity Plasmid-Prep Systems (Invitrogen, Carlsbad, CA, USA). The day before the transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cells were transfected with 0.8 μg of total plasmid DNA per well using LipofectAMINE 2000 (Invitrogen) according to the methods described previously (16). At 48 h after transfection, the cells were used for uptake experiments. To construct a transfectant stably expressing hOCT2, HEK293 cells were transfected with 0.8 μg of total plasmid DNA (pCMV6-XL4: pBK-CMV vector = 2:1) per well. At 24 h after transfection, the cells split between 1:15 and 1:30 were cultured in complete medium containing G418 (0.5 mg/ml) (Wako Pure Chemical, Osaka, Japan). Then 14 to 21 days after transfection, single colonies were picked out. G418-resistant colonies were analyzed by RT-PCR for the expression of hOCT2 mRNA.

Uptake Experiments Using HEK293 Transfectants

Cellular uptake of cationic and anionic compounds using HEK293 cells was measured with monolayer cultures grown

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ABBREVIATIONS: hOCT, human organic cation transporter; GFR, glomerular filtration rate; MPP, 1-methyl-4-phenylpyridinium; NMN, N^1-methylnicotinamide; PAH, *p*-aminohippuric acid; TEA, tetraethylammonium.

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on poly-D-lysine-coated 24-well plates (16). The cells were preincubated with 0.2 ml of incubation medium for 10 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium containing [^{14}C]creatinine, [^{14}C]TEA, [^{14}C]PAH, or [^{3}H]estrone sulfate was added. The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). The composition of high K^+ incubation medium was as follows (in mM): 3 NaCl, 145 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). When indicated, 9.2 mM $BaCl_2$ was added to the incubation medium. The medium was aspirated off at the end of the incubation, and the monolayers were rapidly rinsed twice with 1 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford (20), using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as a standard. For the cis-inhibition study, the uptake of [^{14}C]creatinine was achieved by adding various concentrations of unlabeled inhibitors to the incubation medium. Concentration dependence of creatinine transport by hOCT2 was analyzed using Michaelis-Menten equation; $V = V_{max}[S]/(K_m + [S]) + K_d[S]$, where V is transport rate, V_{max} is the maximal transport rate, [S] is the concentration of creatinine, K_m is Michaelis constant, and K_d is a diffusion constant. The apparent IC_{50} values were calculated from inhibition plots based on the equation, $V = V_0/[1 + (I / IC_{50})^n]$ by a nonlinear least-squares regression analysis with Kaleidagraph Version 3.5 (Synergy Software, Reading, PA, USA) (13). V and V_0 are the uptake of [^{14}C]creatinine in the presence and absence of inhibitor, respectively. I is the concentration of inhibitor, and n is the Hill coefficient.

Materials

[2-^{14}C]Creatinine hydrochloride (55 mCi/mmol) and [ethyl-1-^{14}C] tetraethylammonium (TEA) bromide (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). *p*-[Glycyl-^{14}C]aminohippuric acid (PAH) (50.4 mCi/mmol) and [6,7-^{3}H(*N*)]estrone sulfate ammonium salt (43.5 Ci/mmol) were obtained from Perkin Elmer Life Science Products (Boston, MA, USA). Creatinine, tetraethylammonium bromide, dopamine hydrochloride, guanidine hydrochloride, cimetidine, and (±)-chlorpheniramine maleate were obtained from Nacalai Tesque (Kyoto, Japan). N^1-Methylnicotinamide (NMN) iodide and 1-methyl-4-phenylpyridinium (MPP) iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other compounds used were of the highest purity available.

Statistical Analyses

Data were analyzed statistically by one-way analysis of variance followed by Dunnett's test or non-paired Student's *t* test. p values of less than 0.05 were considered to be significant.

RESULTS

[^{14}C]Creatinine Uptake by HEK293 Cells Expressing Human Organic Ion Transporters

First, we evaluated the uptake of [^{14}C]creatinine by HEK293 cells transfected with hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3 cDNA. As shown in Fig. 1a, the uptake of [^{14}C]creatinine was markedly stimulated in hOCT2-transfected HEK293 cells. In contrast, the uptake of [^{14}C]creatinine by hOCT1-, hOCT2-A-, hOAT1-, and hOAT3-transfected cells was comparable to that by null vector-transfected cells. In these experiments, the functional expression of hOCTs, hOAT1, and hOAT3 in the corresponding batches of the transfected cells was verified by the transport activity of [^{14}C]TEA, [^{14}C]PAH, and [^{3}H]estrone sulfate, respectively (Figs. 1b, 1c and 1d).

Concentration Dependence of [^{14}C]Creatinine Uptake by hOCT2

To examine characteristics of creatinine transport by hOCT2, we constructed HEK293 cells stably expressing hOCT2. Figure 2 shows the concentration-dependence of [^{14}C]creatinine uptake in HEK293 cells stably expressing hOCT2. The uptake of creatinine by these cells was saturated at high concentrations (Fig. 2). The uptake by hOCT2-transfected cells increased time-dependently, and its uptake was linear for up to 2 min (data not shown). The apparent K_m value of the creatinine uptake by hOCT2-transfected cells estimated from three separate experiments using three monolayers was 4.0 ± 0.3 mM. The V_{max} value of the creatinine uptake by hOCT2-transfected cells was 23.5 ± 5.2 nmol·mg protein^{-1}·min^{-1}. Eadie-Hofstee plots were linear (inset of Fig. 2), suggesting absence of endogenous transport system for creatinine in HEK293 cells.

Effect of Membrane Potential on [^{14}C]Creatinine Uptake by hOCT2

Next, we examined the effect of membrane potential on [^{14}C]creatinine uptake by hOCT2-expressing HEK293 cells (Fig. 3). With this approach, increasing the concentration of K^+ in the uptake buffer depolarized the cell membrane potential. The uptake of creatinine decreased in the presence of high K^+ buffer. Furthermore, the accumulation of creatinine decreased in the presence of Ba^{2+}, a nonselective K^+ channel blocker. These results suggest that the transport of creatinine by hOCT2 is dependent on the membrane potential, consistent with the characteristics of hOCT2 (16).

Effect of Organic Cations and Anions on [^{14}C]Creatinine Uptake by hOCT2

To determine the substrate affinity of hOCT2 for cationic compounds, we examined the inhibitory effects of various cationic and anionic compounds on the uptake of creatinine by the hOCT2 transfectants and calculated the apparent IC_{50} values using the equation described in "Materials and Methods" (Fig. 4 and Table I). Cationic drugs (Fig. 4a), neurotoxin and endogenous cations (Fig. 4b) inhibited the uptake of creatinine by the hOCT2 transfectants in a dose-dependent manner. MPP had the most potent inhibitory effect on the uptake of creatinine by hOCT2 among the compounds tested (Table I). Furthermore, hOCT2 showed higher affinities for cationic drugs, H_1- and H_2-receptor antagonists, and endogenous cations, in comparison with the affinity for creatinine. Salicylic acid and PAH had weak inhibitory effects on the uptake of creatinine by hOCT2 at high concentrations (Fig. 4d).

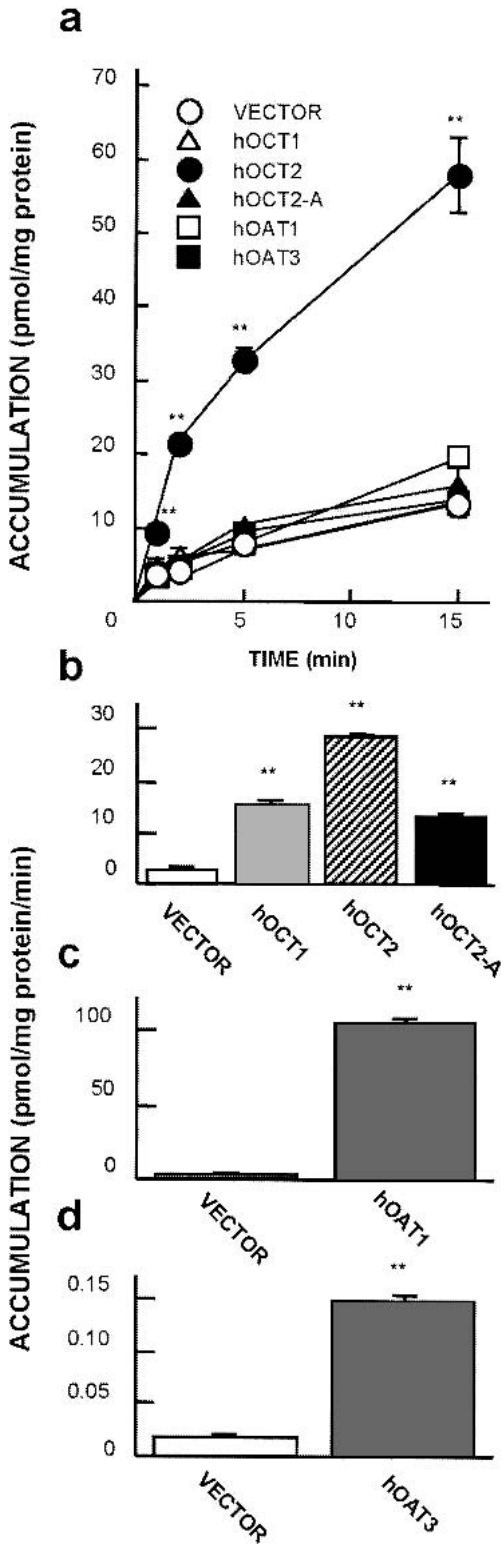

Fig. 1. Transport activity for [^{14}C]creatinine by HEK293 cells transiently expressing human organic ion transporters. (a) HEK293 cells transfected with hOCT1 (△), hOCT2 (●), hOCT2-A (▲), hOAT1 (□), hOAT3 (■), or pCMV6-XL4 vector (○) were incubated for the specified periods at 37°C with 5 μM [^{14}C]creatinine. Each point represents the mean ± SE for three monolayers. (b) HEK293 cells transfected with hOCT1 (shaded column), hOCT2 (hatched column), hOCT2-A (closed column), or null vector (open column) were incubated at 37°C for 1 min with 5 μM [^{14}C]TEA. (c) HEK293 cells transfected with hOAT1 (shaded column), or null vector (open column) were incubated at 37°C for 1 min with 10 μM [^{14}C]PAH. (d) HEK293 cells transfected with hOAT3 (shaded column), or null vector (open column) were incubated at 37°C for 1 min with 19 μM [^{3}H]estrone sulfate. Each column represents the mean ± SE for three monolayers. **$p < 0.01$ vs. null vector-transfected HEK293 cells by Dunnett's test (Figs. 1a and 1b) and Student's *t* test (Figs. 1c and Figs. 1d).

with renal disease, especially in those with glomerular disorders (23–27).

The mechanisms underlying the tubular secretion of creatinine have been controversial; Berglund *et al.* (28), Burgess *et al.* (29), and van Acker *et al.* (30) suggested base-secreting pathways for creatinine secretion based on the findings that concomitant cimetidine or trimethoprim blocked the tubular secretion of creatinine. However, Crawford (31) and Burry and Dieppe (32) demonstrated inhibition of creatinine clearance by exogenous organic anions. Because cimetidine is a good substrate for hOCT2 (13,16,33), and hOCT2 is a predominant organic cation transporter in the human kidney lo-

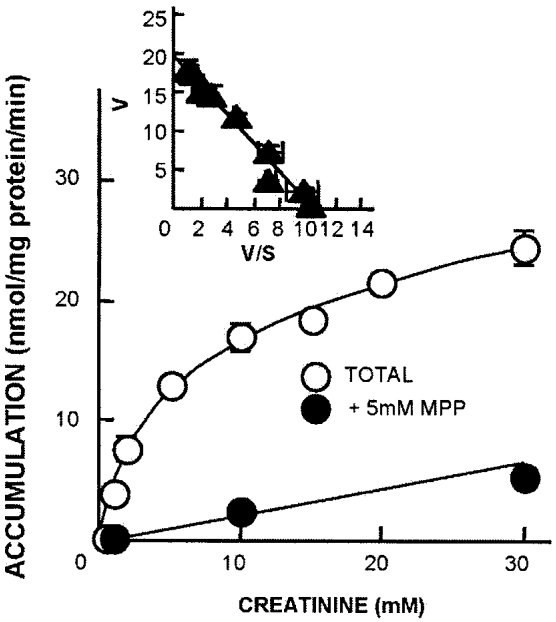

Fig. 2. Concentration-dependence of [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. hOCT2 transfectants were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine in the absence (○) or presence (●) of 5 mM MPP (pH 7.4). Each point represents the mean ± SE for three monolayers from a typical experiment. Inset: Eadie-Hofstee plots of creatinine uptake after a correction for non-saturable components. V, uptake rate (nmol·mg protein^{-1}·min^{-1}); S, creatinine concentration (mM). Unlabeled creatinine was added to [^{14}C]creatinine to give the final concentrations indicated.

DISCUSSION

Creatinine clearance, calculated from serum and urine creatinine concentrations, is often used for the estimation of glomerular filtration rate (GFR). However, creatinine clearance usually exceeds GFR because of the tubular secretion of creatinine (21,22). In addition, overestimation of GFR by means of creatinine clearance has been marked in patients

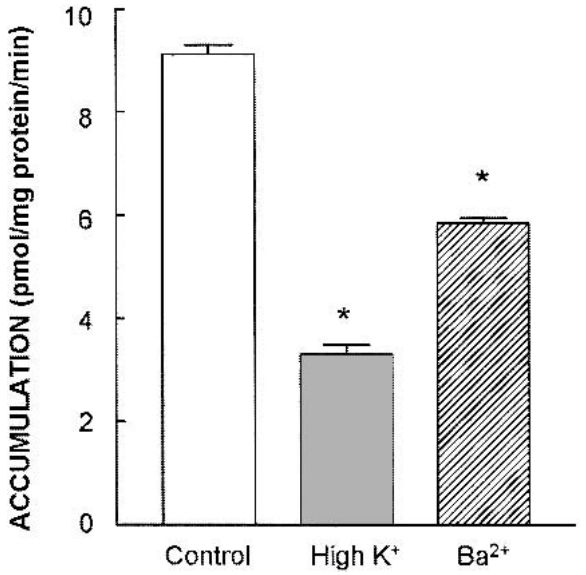

Fig. 3. Effect of membrane potential on [^{14}C]creatinine uptake by HEK293 cells stably expressing hOCT2. HEK293 cells transfected with hOCT2 were incubated with respective buffers at 37°C with 4.5 μM [^{14}C]creatinine. Each column represents the mean ± SE of three monolayers from a typical experiment. **$p < 0.01$ vs. control by Dunnett's test.

calized at the basolateral membranes of the proximal tubules (17), we supposed hOCT2 to be a responsible transporter mediating tubular secretion of creatinine. In the current study, hOCT2 was the only transporter mediating creatinine transport among several organic ion transporters examined (Fig. 1), suggesting hOCT2 to be the responsible transporter regulating creatinine uptake at the basolateral membranes of renal proximal tubules. We also found much higher Michaelis constant of creatinine for hOCT2 (K_m: 4.0 ± 0.3 mM) than physiological (about 45–85 μM for male and 30–60 μM for female) and even pathophysiological concentrations of creatinine in human serum, suggesting that hOCT2 could function as creatinine transporter without saturation. We speculate that this low affinity transport of creatinine by hOCT2 would be beneficial for the efficient extrusion of creatinine from circulation even in the patients with decreased glomerular filtration.

In general, organic ion transporters are multispecific (polyspecific) and thereby share common substrates. In the current study, however, we found that creatinine is specifically transported by hOCT2, but not by any other organic cation and anion transporters examined. To our knowledge, this is the first demonstration that creatinine, an endogenous organic cation, is a specific substrate for hOCT2. Because hOCT1 is dominantly expressed in the liver, but not in the kidney (18,19), it is reasonable that renal hOCT2 would regulate the kidney-specific secretion of creatinine.

Several reports have emerged to date that cimetidine inhibits the tubular secretion of creatinine in humans without altering GFR (29,30). Unlike cimetidine, ranitidine, another H_2-receptor antagonist, does not inhibit the tubular secretion of creatinine (34). The therapeutic range of cimetidine is about 6- to 10-fold higher than that of ranitidine, and 20- to 50-fold higher than that of famotidine (35). In the current study, the order of the affinity of H_2-receptor antagonists for

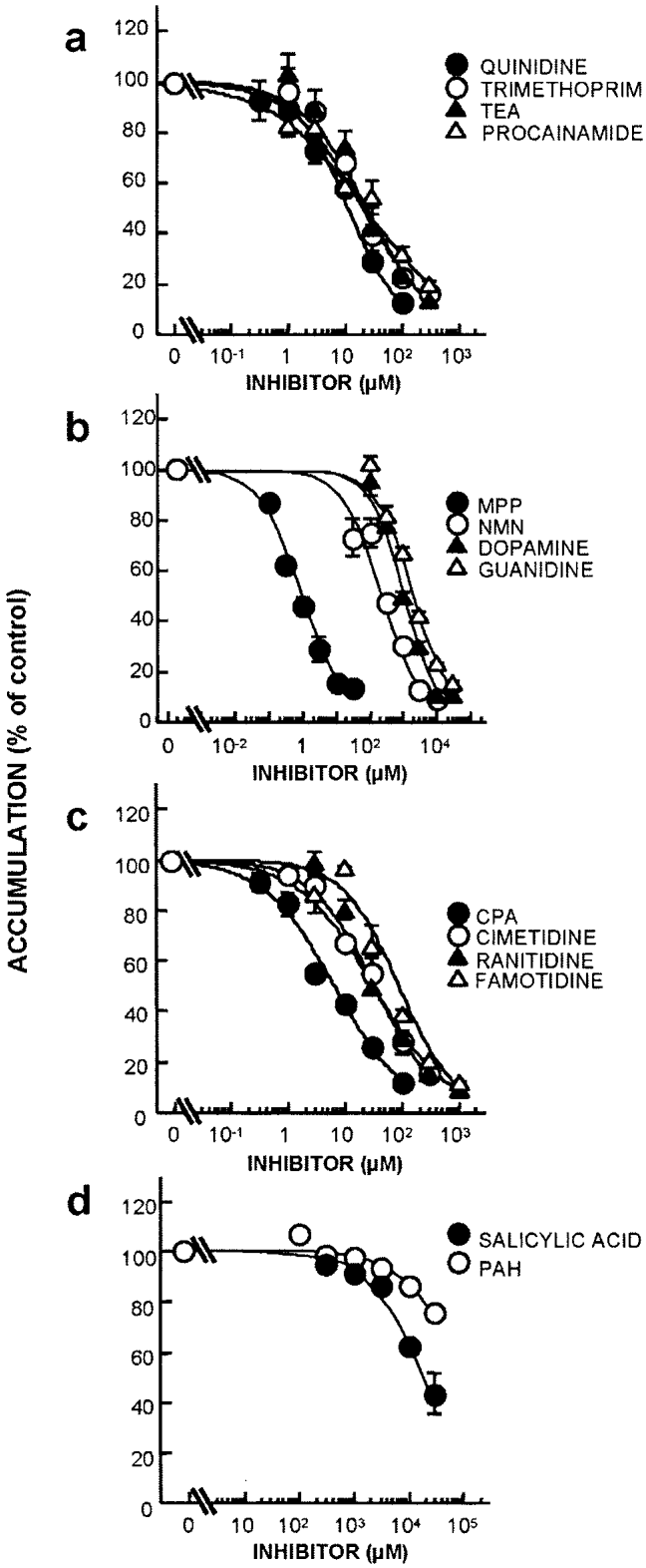

Fig. 4. Effects of cationic and anionic compounds on [^{14}C]creatinine uptake by the hOCT2-transfectants. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine (pH 7.4) in the presence of (a) quinidine (●), trimethoprim (○), TEA (▲), or procainamide (△); (b) MPP (●), NMN (○), dopamine (▲), or guanidine (△); (c) chlorpheniramine (CPA) (●), cimetidine (○), ranitidine (▲), or famotidine (△); (d) salicylic acid (●) or PAH (○). Each point represents the mean ± SE for three monolayers from a typical experiment.

Table I. The Apparent IC_{50} Values of Various Cationic and Anionic Compounds for [^{14}C]Creatinine Uptake by hOCT2

Compounds	Apparent IC_{50} values for [^{14}C]creatinine uptake (μM)
MPP	1.1 ± 0.2
Chlorpheniramine	6.0 ± 0.3
Quinidine	10 ± 1
Trimethoprim	21 ± 2
TEA	24 ± 6
Cimetidine	27 ± 6
Procainamide	28 ± 10
Ranitidine	38 ± 5
Famotidine	70 ± 8
NMN	310 ± 70
Dopamine	1400 ± 100
Guanidine	2200 ± 100
Salicylic acid	14000 ± 3000

See experimental conditions in the legend of Fig. 4. The apparent IC_{50} values were calculated from inhibition plots (Fig. 4) by nonlinear regression analysis as described in "Materials and Methods." The data represent the mean ± SE for three independent experiments. MPP, 1-methyl-4-phenylpyridinium; TEA, tetraethylammonium; NMN, N′-methylnicotinamide.

the uptake of creatinine by hOCT2 was cimetidine ~ ranitidine > famotidine (Fig. 4C and Table I). These findings indicate that at therapeutic concentrations, cimetidine would moderately inhibit creatinine uptake via hOCT2, whereas ranitidine and famotidine would exert almost no influence. We speculate that the stronger inhibitory effect of cimetidine on the tubular secretion of creatinine is likely to be associated with the high affinity binding of cimetidine to hOCT2 as well as the higher therapeutic range of cimetidine compared with other H_2-receptor antagonists.

In conclusion, hOCT2 mediates basolateral membrane transport of creatinine in the human kidney. Unlike hOCT1, hOCT2 should be responsible for the kidney specific disposition of creatinine.

ACKNOWLEDGMENTS

This work was supported in part by the Smoking Research Foundation, by a grant-in-aid for Scientific Research on Human Genome, Tissue Engineering and Food Biotechnology from Ministry of Health, Labor and Welfare of Japan (H12-Genome-019), by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan, and by the 21st Century COE program "Knowledge Information Infrastructure for Genome Science."

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