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The Cation Transporters rOCT1 and rOCT2 Interact with Bicarbonate but Play Only a Minor Role for Amantadine Uptake into Rat Renal Proximal Tubules

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ABSTRACT

In renal proximal tubules, the organic cation transporters rOCT1 and rOCT2 are supposed to mediate the first step in organic cation secretion. We investigated whether previously described differences in amantadine and tetraethylammonium (TEA) uptake into isolated renal proximal tubules could be explained by differences in their transport by rOCT1 and rOCT2. By expressing rOCT1 and rOCT2 in *Xenopus* oocytes and HEK 293 cells, we demonstrated that both transporters translocated amantadine. In *Xenopus* oocytes, the inhibitory potency of several rOCT1/2 inhibitors was similar for amantadine compared to TEA uptake and supports amantadine transport by rOCT1 and rOCT2. In proximal tubules, procainamide, quinine, cyanine$_{863}$, choline, and guanidine in concentrations that inhibit rOCT1/2-mediated TEA or amantadine uptake in *Xenopus* oocytes exhibited no effect on amantadine uptake. At variance, these inhibitors blocked TEA uptake into proximal tubules. Amantadine and TEA transport were sensitive to modulation by 25 mM bicarbonate. The effect of bicarbonate on organic cation transport was dependent on substrate (amantadine or TEA), cell system (oocytes, HEK 293 cells, or proximal tubules), and transporter (rOCT1 or rOCT2). In proximal tubules, only amantadine uptake was stimulated by bicarbonate. The data suggested that rat renal proximal tubules contain an organic cation transporter in addition to rOCT1 and rOCT2 that mediates amantadine uptake and requires bicarbonate for optimal function. TEA uptake by the basolateral membrane may be mediated mainly by rOCT1 and rOCT2, but these transporters may be in a different functional or regulatory state when expressed in cells or oocytes compared with expression in vivo.

Several organic cation transporters (rOCT1, rOCT1a, rOCT2, and rOCT3) have been molecularly identified, have been shown to be expressed in the rat kidney, and have been demonstrated to transport the prototypical organic cation TEA when expressed in cell lines and/or *Xenopus* oocytes (Grundemann et al., 1994; Okuda et al., 1996; Gorboulev et al., 1997; Zhang et al., 1997; Kekuda et al., 1998). The majority of transport, in situ hybridization, and immunohistochemical evidence indicates that rOCT1 and rOCT2 are basolateral membrane transporters and are responsible for the first step in organic cation secretion in the proximal tubule (Grundemann et al., 1994; Urakami et al., 1998; Budiman et al., 2000; Karbach et al., 2000; Sugawara-Yokoo et al., 2000; Sweet et al., 2000). OCT3 mRNA expression has been detected in mouse proximal and distal tubules, but its assignment to the apical or basolateral membrane has not been determined (Wu et al., 2000). Studies on isolated OCTs have been numerous and support their proposed physiological role in the kidney (renal secretion of organic cations), but sufficient in vivo data are lacking to validate the relative contribution of the individual OCTs to renal secretion of TEA or clinically important cations. With exception, OCT1-deficient mice have been recently generated and interestingly have higher urinary excretion rates for TEA than control mice (Jonker et al., 2001). In this study, organic cation transport assays were performed in rOCT1- and rOCT2-expressing human embryonic kidney (HEK 293) cells, *Xenopus laevis* oocytes, and a model that more closely represents the normal renal tubule environment (isolated proximal tubules). By

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ABBREVIATIONS: OCT, organic cation transporter; TEA, tetraethylammonium; rOCT, rat organic cation transporter; hOCT, human organic cation transporter; KHS, Krebs-Henseleit solution; CT, Cross-Taggart; ANOVA, analysis of variance; HSD, honestly significant difference.

comparing the kinetics of organic cation transport in cells versus tubules under different conditions, the role of the individual OCTs in mediating renal tubule secretion of TEA and other organic cation drugs may be more clearly defined.

Previously, it was reported that amantadine and TEA characterize distinct basolateral membrane transport mechanisms in renal tubules (Goralski and Sitar, 1999). Transport sites for amantadine in proximal tubules can be subdivided into bicarbonate-dependent (high-affinity, high-capacity) sites responsible for most of the amantadine uptake and less efficient bicarbonate-independent (low-affinity, low-capacity) sites (Escobar et al., 1994; Escobar and Sitar, 1995). At variance, TEA uptake into proximal tubules was independent of bicarbonate and TEA did not inhibit amantadine uptake (Goralski and Sitar, 1999). Given that TEA is transported by rOCT1 and rOCT2, we hypothesized that amantadine uptake via rOCT1 and rOCT2 is minimal compared with TEA and that amantadine uptake is representative of a novel type of OCT(s) that is (are) important in mediating renal tubule secretion of certain organic cations. Amantadine transport by rOCT1 and rOCT2 has not yet been studied in single transporter-expressing cells. It has been demonstrated, however, that amantadine generates inward cation currents in *Xenopus* oocytes that express the human organic cation transporter hOCT2 (Busch et al., 1998). To determine whether rOCT1 and rOCT2 contribute to amantadine transport, amantadine uptake into HEK 293 cells and *Xenopus* oocytes expressing rOCT1 and rOCT2 was examined. Second, the ability of substrates or inhibitors of rOCT1 and rOCT2 to block amantadine and TEA uptake into the renal tubule or cell preparations was evaluated and compared. Of the compounds chosen, cyanine$_{863}$, procainamide, quinine, guanidine, dopamine, and corticosterone have been reported to have substantially different K_M or IC_{50} values for the inhibition of TEA uptake into rOCT1 or rOCT2-expressing *Xenopus* oocytes (Koepsell, 1998; Okuda et al., 1999; Arndt et al., 2001). Comparing IC_{50} values for the inhibitors in cells expressing a single transporter versus isolated tubules and with different buffers (bicarbonate versus nonbicarbonate) may help determine whether differences in rOCT1- and/or rOCT2-mediated transport contribute to amantadine and TEA transport differences observed in the proximal tubule.

Materials and Methods

Amantadine Transport and Electrical Measurements in *Xenopus laevis* Oocytes. The methodology for OCT transfection and expression in *Xenopus* oocytes has been previously described in detail (Busch et al., 1998; Arndt et al., 2001). Tracer uptake of 10 μM [^{3}H]amantadine was measured after 3 days expression in oocytes that were injected with 10 ng/oocyte of rOCT1 RNA or rOCT2 RNA. The uptake measurements were performed after 1 h incubation with Ori buffer [5 mM 3-(-*N*-morpholino)propanesulfonic acid-NaOH, pH 7.4, 100 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$] without inhibitor or with 100 μM cyanine$_{863}$. Electrical measurements were performed as previously described (Arndt et al., 2001). For the electrical measurements, quinine was used as inhibitor instead of cyanine$_{863}$ because cyanine$_{863}$ generates a nonspecific electrical effect. Krebs-Henseleit solution (KHS) was the buffer used for amantadine and TEA transport studies that were performed in the presence of bicarbonate. KHS (pH 7.4) contained: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose. For all transport assays in KHS, the pH and pCO_2 levels of the buffer were adjusted by bubbling with O_2/CO_2 (95%/5%).

Transport Measurements in rOCT1- and rOCT2-Expressing Cells. Generation of the HEK 293 cell line stably transfected with rOCT1 has been reported (Busch et al., 1996). HEK 293 cells expressing rOCT2 have been generated in the same way. Nontransfected HEK 293 cells (American Tissue Culture Collection, Manassas, VA) and HEK 293 cells stably transfected with rOCT1, rOCT2, or the empty plasmid vector (pRc-CMV; Invitrogen, Groningen, The Netherlands) were grown to 80% confluence in 175-ml culture flasks. Before assays, each flask was rinsed twice with KHS (10 ml). Cells were detached by shaking with 10 ml of buffer followed by centrifugation at 1000*g* for 11 min. The pelleted cells were resuspended in 1.4 ml of KHS and placed in a water bath (25°C) until ready for use. Cells (80 μl, final protein content 5–6 mg/ml as measured by the Biuret assay) were placed in microcentrifuge tubes at 25°C with shaking. [^{14}C]TEA (10 μl, 20 μM final concentration) or [^{3}H]amantadine (10 μM final concentration) was added to the wall of the centrifuge tube. The transport reaction was started by vortexing and placed in a water bath for 3 s. At the end of 3 s, 1 ml of ice-cold stopping buffer [(10 μM quinine in KHS or Cross-Taggart (CT) buffer] was added to terminate the reaction. Cells were pelleted by centrifuging for 1 min at 13,000*g*, washed twice with 1 ml of ice-cold stopping buffer, and dissolved in 200 μl of Triton X-100 (0.1% v/v). Radioactivity was determined by scintillation counting in a Beckman model LS5801 scintillation counter (Beckman Coulter, Inc., Fullerton, CA).

Renal Tubule Preparation. The experimental procedures involving the use of animals have been approved by the University of Manitoba Protocol Management and Review Committee. Male Sprague-Dawley rats (250g-300g) were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.); a midline incision was made, and animals were killed by cutting the aorta. Immediately following sacrifice, both kidneys were removed, decapsulated, and placed in ice-cold KHS. Separation of proximal tubules was performed by a modified Percoll density gradient centrifugation method previously reported in detail by our laboratory (Wong et al., 1990; Escobar et al., 1994; Goralski and Sitar, 1999). The purity of the proximal tubule fraction was assessed by measuring levels of the enzyme marker alkaline phosphatase and by microscopic examination, as previously reported (Wong et al., 1991). If the transport assays included measurements in the absence of bicarbonate, the final resuspension of the tubule fragments would be done with CT phosphate buffer instead of KHS. CT contained 135 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.4 mM KH_2PO_4, 15 mM sodium phosphate buffer (pH 7.4), 1.0 mM $CaCl_2$, 11 mM glucose. Tissue protein was determined before the transport assays by the Biuret method (Gornall et al., 1949) and was adjusted with KHS or CT to give a final protein concentration of 6 to 8 mg/ml.

Transport Measurements in Isolated Renal Tubules. The effects of known substrates or inhibitors of rOCT1 and rOCT2 (guanidine, choline, procainamide, dopamine, cyanine$_{863}$, quinine, and corticosterone) and OCTN2 (carnitine) on the uptake of 10 or 20 μM [^{14}C]TEA and 10 μM [^{3}H]amantadine in proximal tubules in the presence and absence of bicarbonate were evaluated. With the exception of corticosterone (which was dissolved in 95% v/v ethanol), all stock drug solutions were made up in distilled water. The final concentration of ethanol in the reaction mixture was 2% v/v and did not affect control amantadine or TEA uptake (data not shown). [^{3}H]amantadine (30 s) and [^{14}C]TEA (60 s) uptake assays were performed as previously described (Goralski and Sitar, 1999). The uptake reactions were terminated by the addition of 2 × 4 ml of ice-cold KHS, followed by rapid filtration under negative pressure, through glass filters (32; Schleicher and Schuell, Inc., Keene, NH). The filters were placed in vials containing 4 ml of Ready Safe scintillation fluid (Beckman Instruments, Inc.) and counted in a Beckman model LS5801 scintillation counter. Nonspecific uptake to tissue and filters was determined by measuring uptake of amantadine

(30 s) or TEA (60 s) containing a saturating amount of unlabeled amantadine (10 mM) or TEA (10 mM), respectively.

Data Analysis. For the individual experiments, each data point for the uptake and inhibition studies was replicated in triplicate. Data are expressed as mean ± S.E.M. of at least four experiments unless otherwise stated. For amantadine and TEA uptake into isolated proximal tubules in the presence of increasing inhibitor concentrations, the data are expressed as a percentage of control uptake in the respective buffers. For each buffer (KHS or CT), 100% control uptake was defined as the rate of 10 μM amantadine or TEA uptake in the absence of any inhibitors in the medium. IC_{50} values were determined from the inhibition profiles by regressive probit analysis of increasing inhibitor concentrations (Cheng and Prusoff, 1973). Due to the unequal variances for the amantadine and TEA groups, the IC_{50} values were transformed for statistical analysis. Each IC_{50} was transformed by $\log_{10}$ ($IC_{50} \times 10$), which resulted in similar variances between the two groups. IC_{50} values were first multiplied by 10 to remove the possibility of negative numbers after the $\log_{10}$ transformation. For uptake and inhibition studies in isolated proximal tubules, an ANOVA model with data grouped according to buffer (KHS or CT) and substrate (amantadine or TEA) was used for statistical comparison of the transformed IC_{50} data for each inhibitor. For uptake and inhibition studies in cells, a two-way ANOVA with data grouped according to cell type (nontransfected, empty vector-transfected, and OCT1-expressing or rOCT2-expressing) and buffer (KHS and CT) was used for statistical comparisons. Multiple comparisons of the significant ANOVA were performed by Tukey's HSD test. Differences between means with a P value of 0.05 or less were considered to be significant.

Chemicals. [^{3}H]Amantadine (28 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK). [^{14}C]TEA (55 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Collagenase was obtained from Roche Diagnostics (Laval, QC, Canada). Unlabeled amantadine was obtained from DuPont Canada, Inc. (Mississauga, ON, Canada). Choline, carnitine, guanidine, dopamine, corticosterone, procainamide, cyanine$_{863}$, and quinine were all obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade available from commercial suppliers.

Results

rOCT1 and rOCT2 Mediate Amantadine Uptake into *Xenopus laevis* Oocytes. In both rOCT1- and rOCT2-injected oocytes, uptake of 10 μM [^{3}H]amantadine was greater than in water-injected oocytes (Fig. 1A). Amantadine uptake was inhibited by 100 μM cyanine$_{863}$ in OCT1- and OCT2-injected oocytes but not in the control oocytes. Choline, a transported substrate of rOCT1 and rOCT2 was used as a control substrate in electrophysiological measurements. In control oocytes that were not injected with cRNA, no significant currents were induced after superfusion with amantadine, choline, or quinine (Fig. 1B). In rOCT1- and rOCT2-injected oocytes, choline and amantadine induced inwardly directed cation currents (Fig. 1, C and D). The amantadine-induced currents could be blocked by 100 μM quinine. These data provide the first direct evidence of amantadine transport by rOCT1 and rOCT2.

Inhibitor Sensitivity of Amantadine Uptake by rOCT1 or rOCT2 Expressed in *Xenopus* Oocytes. We attempted to determine whether inhibitor sensitivity of rOCT1- and rOCT2-mediated amantadine transport was similar to TEA (Fig. 2). We used inhibitor (quinine, cyanine$_{863}$, procainamide, choline, guanidine, and TEA) concentrations that were in range of the IC_{50} values determined for

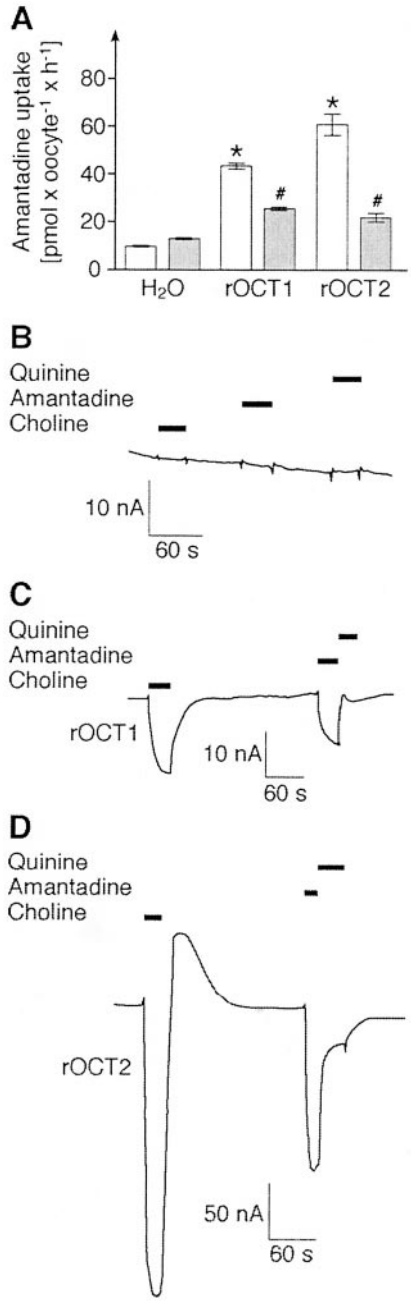

Fig. 1. A, uptake of 10 μM [^{3}H]amantadine (picomoles per oocyte per hour) was measured after 3 days expression in *Xenopus* oocytes that were injected with 10 ng/oocyte of rOCT1 RNA or rOCT2 RNA. The uptake measurements were performed after 1 h incubation with Ori buffer (pH 7.4) [5 mM 3-(-*N*-morpholino)propanesulfonic acid-NaOH, 100 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$] without inhibitor (open bars) or with 100 μM cyanine$_{863}$ (shaded bars). Bars represent the mean ± S.E.M. of 10 oocytes. ⋆, $P \leq 0.001$ compared with uptake in water injected control oocytes; #, $P < 0.05$ compared with uptake in the absence of cyanine$_{863}$ (unpaired t tests). Electrical measurements in water (B), rOCT1 (C), and rOCT2 (D) injected *Xenopus* oocytes were performed as previously described (Arndt et al., 2001). The traces are representative for three individual measurements. The oocytes were clamped at −50 mV. They were superfused first with Ori buffer, then with Ori buffer containing 10 mM choline, then again with Ori buffer, then with Ori buffer containing 200 μM of amantadine, then Ori buffer containing 100 μM of quinine, and finally with Ori buffer.

the inhibition of TEA uptake by rOCT1 or by rOCT2 (Arndt et al., 2001) or for inhibition of amantadine uptake by proximal tubules (Table 1). Two micromolar and 1 mM of cya-

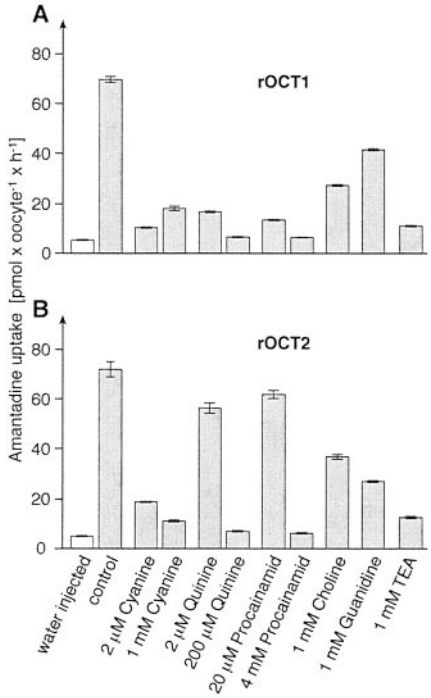

Fig. 2. Inhibition of amantadine uptake expressed in *Xenopus* oocytes by (A) rOCT1 or (B) rOCT2. The oocytes were injected and incubated for expression and uptake measurements as in Fig. 1. For uptake measurements, the oocytes were incubated for 1 h at room temperature with 10 μM [^{3}H]amantadine in the presence of the indicated inhibitor concentrations. Means ± S.E.M. from 8 to 10 measurements are shown. White bars represent amantadine uptake with oocytes of the same batch that were not injected.

TABLE 1

IC_{50} values (micromolar) for inhibition of renal proximal tubule uptake of 10 μM [^{3}H]amantadine and 10 μM [^{14}C]TEA

Inhibition studies were performed in the presence (KHS) or absence (CT) of 25 mmol/l bicarbonate at pH 7.4. Transport assay duration was 30 s for amantadine and 60 s for TEA. IC_{50} values are reported as mean ± S.E.M. of four or five individual experiments. The IC_{50} data were transformed by the factor [$\log_{10}(IC_{50} \times 10)$] to produce similar variance in the amantadine and TEA groups for two-way ANOVA followed by Tukey's HSD test.

Inhibitor		Proximal	
		KHS	CT
$Cyanine_{863}$	AM	930 ± 300*	880 ± 170*
	TEA	1.6 ± 0.8	1.0 ± 0.3
Quinine	AM	180 ± 20*	350 ± 90*
	TEA	1.6 ± 0.2	1.6 ± 0.4
Procainamide	AM	2200 ± 800*	6400 ± 2000*
	TEA	19 ± 7	42 ± 17
Dopamine	AM	DNI	DNI
	TEA	490 ± 60	300 ± 100
Corticosterone	AM	DNI	DNI
	TEA	4.9 ± 1.9	3.0 ± 0.7

AM, amantadine; DNI, did not inhibit.
* $P < 0.001$, inhibition of amantadine transport versus TEA transport within buffer group.

$nine_{863}$ inhibited more than 80% of amantadine uptake by rOCT1 or rOCT2. Quinine (2 μM) and procainamide (20 μM) inhibited 80% of rOCT1 but only about 20% of rOCT2-mediated amantadine uptake. Higher concentrations of quinine (200 μM) and procainamide (4 mM) inhibited similar amounts (90%) of amantadine uptake by rOCT1 and rOCT2. This inhibitor sensitivity corresponds to the IC_{50} values for $cyanine_{863}$ (0.5 and 2.5 μM), quinine (4.1 and 23 μM), and procainamide (20 and 445 μM) that were previously determined for inhibition of TEA transport by rOCT1 and rOCT2, respectively (Arndt et al., 2001). The data suggest that the inhibitor sensitivity of rOCT1 and rOCT2 expressed in *Xenopus* oocytes does not depend on the transported substrates (i.e., amantadine versus TEA). The observation that amantadine uptake by rOCT1 and rOCT2 is inhibited more than 90% by 1 mM TEA and between 60 and 70% by 1 mM choline and guanidine can be explained by a lower affinity of choline and guanidine compared with TEA.

Bicarbonate Modulation of rOCT1- and rOCT2-Mediated Organic Cation Transport in *Xenopus* Oocytes. We evaluated whether rOCT1- and rOCT2-mediated transport of amantadine and TEA was sensitive to modulation by 25 mM bicarbonate. [^{3}H]Amantadine (2.5 μM) and [^{14}C]TEA (10 μM) uptake into rOCT1- and rOCT2-expressing *Xenopus* oocytes were determined in the nonbicarbonte Ori buffer or 25 mM bicarbonate containing KHS at pH 7.4. Figure 3 shows that amantadine uptake by rOCT1 or rOCT2 was similar in bicarbonate-free Ori buffer compared with bicarbonate-containing KHS buffer, whereas TEA uptake by both transporters was significantly reduced in KHS buffer. To elucidate whether the apparent inhibitory effect of bicarbonate on TEA uptake expressed by rOCT1 and rOCT2 reflects a change of

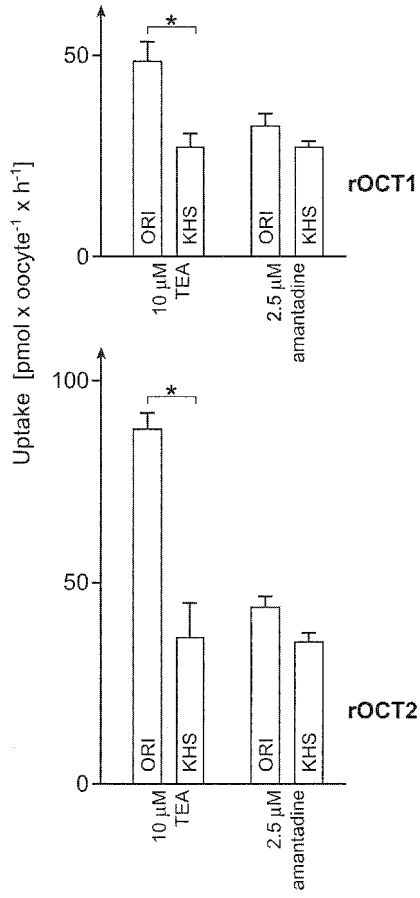

Fig. 3. Bicarbonate modulation of [^{3}H]amantadine (2.5 μM) and [^{14}C]TEA (10 μM) uptake into rOCT1-expressing (A) or rOCT2-expressing (B) *Xenopus* oocytes. The oocytes were injected and incubated for expression and uptake measurements as in Fig. 1. Tracer uptake experiments were carried out in Ori buffer (no bicarbonate) or KHS (25 mM bicarbonate) at pH 7.4. Each bar represents the mean ± S.E.M. from nine measurements. ⋆, $P < 0.05$ compared with TEA uptake in Ori buffer (unpaired t test).

affinity or maximal transport rate, we clamped oocytes expressing rOCT1 or rOCT2 at −50 mV and superfused them with saturating concentrations of amantadine (200 μM), TEA (1 mM), or choline (10 mM) in the presence (KHS) or absence (Ori buffer) of 25 mM bicarbonate. The inwardly directed cation currents that were induced after superfusion of the oocytes with these cations were not significantly different in the two buffers (data not shown). This observation suggests that the apparent effects of bicarbonate observed in the radioactive uptake measurements are due to bicarbonate-dependent changes of K_M values or to bicarbonate-dependent changes of the membrane potential.

rOCT1- and rOCT2-Expressed Amantadine and TEA Uptake by HEK 293 Cells. We used HEK 293 cells stably transfected with rOCT1 and rOCT2 to compare effects of bicarbonate on transport of amantadine and TEA in a different expression system. With these cells, we performed transport assays in 25 mM bicarbonate containing buffer (KHS; pH 7.4) or in a nonbicarbonate buffer (CT; pH 7.4). In Fig. 4, it is demonstrated that amantadine uptake into rOCT1- and rOCT2-expressing HEK 293 cells was saturable and linear for approximately 3 s when assays were carried out in CT buffer. When assays were carried out in KHS buffer, amantadine uptake into rOCT1 and rOCT2 cells was also linear for approximately 3 s (data not shown). In KHS buffer, both rOCT1 and rOCT2 increased the 3 s uptake of 10 μM [^{3}H]amantadine compared with HEK 293 cells that were transfected with the empty vector (Fig. 5A). In CT buffer, the 3-s uptake of 10 μM amantadine into HEK 293 cells was increased after transfection with rOCT2 but not with rOCT1 (Fig. 5B). In KHS buffer, amantadine uptake was inhibited about 60% by 200 μM quinine in rOCT1- and rOCT2-expressing, but not in the empty vector-transfected, HEK 293 cells (Fig. 5A). In CT buffer, rOCT2-expressed uptake of amantadine was inhibited (70%) by 200 μM quinine, whereas amantadine uptake into rOCT1 or empty vector-transfected control cells was not inhibited (Fig. 5B). Since amantadine uptake in control cells could be inhibited by higher quinine concentrations (IC_{50} = 590 ± 60 μM and 440 ± 80 μM in KHS and CT buffer, respectively), it may be mediated by an endogenous transporter.

Contrary to amantadine, no significant endogenous uptake of TEA was detected in HEK 293 cells (data not shown). Figure 6 shows uptake of 20 μM TEA that was measured in the rOCT1- and rOCT2-transfected cells. TEA uptake was greater in rOCT2-expressing cells compared with rOCT1-expressing cells when assays were performed in the presence

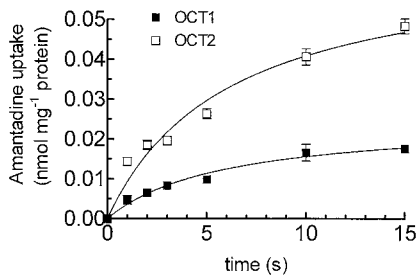

Fig. 4. Representative plots showing amantadine (10 μM) uptake versus time into HEK 293 cells expressing rOCT1 and rOCT2. Assays were performed in CT buffer at pH 7.4. Amantadine uptake is expressed as nanomoles per milligram of protein. Data points represent the mean ± S.E.M. from four measurements.

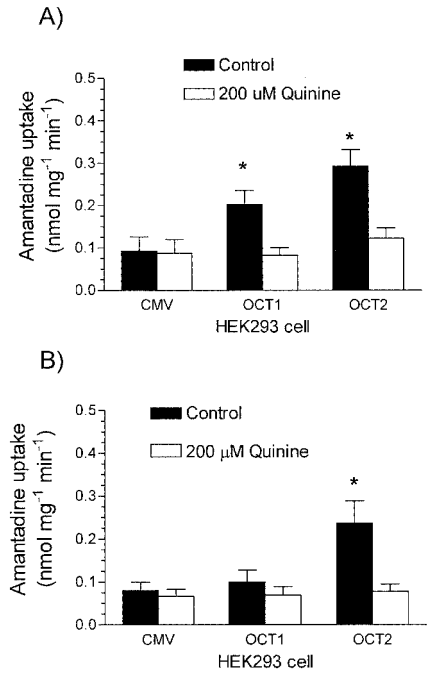

Fig. 5. [^{3}H]Amantadine (10 μM) uptake into empty vector-transfected (CMV), rOCT1- and rOCT2-expressing HEK 293 cells in the presence and absence of 200 μM quinine. Transport assays were 3-s in duration and were performed in KHS buffer (A) or CT buffer (B). KHS buffer contained 25 mM bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). CT buffer (pH 7.4) was used as the nonbicarbonate-containing buffer for transport assays. The rates of amantadine uptake are expressed as nanomoles per milligram of protein per minute, and each bar represents the mean ± S.E.M. of four to six separate measurements. ⋆, $P < 0.05$ compared with amantadine uptake in the presence of 200 μM quinine within cell group (unpaired t test).

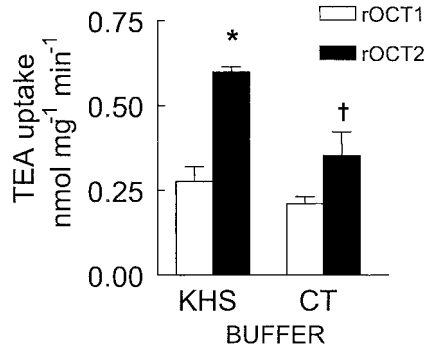

Fig. 6. Rates (nanomoles per milligram of protein per minute) of 20 μM [^{14}C]TEA transport (3 s) into rOCT1- and rOCT2-transfected HEK 293 cells. TEA uptake was not observed in the control cells and was thus omitted from the figure. TEA uptake measurements were carried out in the KHS or CT buffer at pH 7.4. KHS buffer contained 25 mM bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). Each symbol represents the mean ± S.E.M. of at least five separate determinations. ⋆, $P < 0.05$ higher than TEA uptake by rOCT1-expressing cells in KHS; †, $P < 0.05$ lower compared with rOCT2-mediated TEA uptake in KHS buffer (two-way ANOVA followed by Tukey's HSD test).

of bicarbonate. In contrast to the data obtained with *Xenopus* oocytes, in HEK 293 cells, TEA uptake by rOCT2 was higher in KHS compared with CT buffer. TEA uptake into HEK 293 cells expressing rOCT1 and rOCT2 was inhibited by amantadine, cyanine$_{863}$, quinine, and procainamide (Fig. 7; Table 2). The level of transport inhibition by these compounds depended on the expressed type of transporter (rOCT1 versus

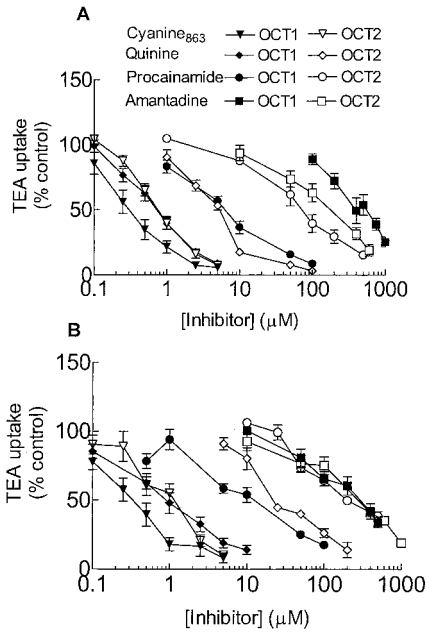

Fig. 7. Inhibition of [^{14}C]TEA (20 μM) uptake (3 s) into rOCT1-expressing HEK 293 cells (shaded symbols) and rOCT2-expressing HEK 293 cells (open symbols) by cyanine$_{863}$ (triangles), quinine (diamonds), procainamide (circles), and amantadine (squares) in KHS (A) or CT (B) buffer at pH 7.4. Data are represented as a percentage of control TEA uptake in the absence of inhibitors. KHS buffer contained 25 mM^{-1} bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). Each symbol represents the mean ± S.E.M. of three to six separate determinations.

rOCT2) and buffer (KHS or CT). At variance to rOCT1, TEA uptake mediated by rOCT2 displayed decreased sensitivity to inhibition by quinine or procainamide in the absence of bicarbonate (i.e., IC_{50} increased in CT compared with KHS). Inhibition of TEA uptake via rOCT1 and rOCT2 by amantadine or cyanine$_{863}$ was not dependent on the presence of bicarbonate in the buffer. TEA uptake by rOCT2 was more potently inhibited by amantadine compared with TEA uptake by rOCT1 in the presence but not in the absence of bicarbonate. At variance, quinine and procainamide were more potent inhibitors of TEA uptake via rOCT1 compared with rOCT2 in the presence and absence of bicarbonate. In the absence of bicarbonate, however, the transporter difference (rOCT1 > rOCT2) in sensitivity to these inhibitors was more pronounced. The sensitivity of rOCT1- and rOCT2-expressing HEK 293 cells to some inhibitors depended on the transported substrates (amantadine versus TEA). Specifically, cyanine$_{863}$ inhibited amantadine uptake into OCT1- and OCT2-expressing HEK 293 cells with less potency than inhibition of TEA uptake (Table 2).

Amantadine but Not TEA Uptake into Isolated Renal Proximal Tubules is Bicarbonate-Dependent. The control rates of 10 μM amantadine and 10 μM TEA uptake into isolated renal proximal tubules in the absence of inhibitors are shown in Fig. 8. In contrast to transport expressed in *Xenopus* oocytes by rOCT1 or rOCT2 and consistent with previous data, the uptake of amantadine into proximal tubules was 3-fold greater in the bicarbonate-containing medium as opposed to the nonbicarbonate medium. Unlike in rOCT1/2-expressing *Xenopus* oocytes or rOCT2-expressing HEK 293 cells, TEA (10 μM) uptake into proximal tubules was similar in the presence and absence of bicarbonate. The rates of 10 μM amantadine uptake into isolated proximal tubules were 5 (CT) to 10 (KHS) times greater than those reported for TEA.

Inhibitors of rOCT1 and rOCT2 More Potently Inhibit TEA Versus Amantadine Uptake Into Isolated Renal Proximal Tubules. The effect of inhibitors or substrates of rOCT1 and rOCT2 on amantadine and TEA uptake into isolated proximal tubules is shown in Fig. 9A–E. Cyanine$_{863}$, quinine, procainamide, dopamine, and corticosterone all selectively inhibited TEA over amantadine transport into proximal tubules in KHS and CT. Dopamine (2 mM) and corticosterone (500 μM) did not inhibit amantadine transport into proximal tubules in KHS and CT (Fig. 9, D and E). Calculated IC_{50} values for inhibition of amantadine and TEA uptake are shown in Table 1. The data show that TEA uptake was more potently inhibited than amantadine uptake in KHS and CT: 600- to 900-fold by cyanine$_{863}$ ($P < 0.001$), 100- to 200-fold by quinine ($P < 0.001$), and 100- to 150-fold by procainamide ($P < 0.001$). Unlike in rOCT2-expressing HEK 293 cells, which displayed bicarbonate-dependent inhibition, in proximal tubules all compounds inhibited TEA transport similarly in the presence and absence of bicarbonate. For quinine and procainamide inhibition of amantadine transport, there was a trend toward increasing IC_{50} in CT compared with KHS. This phenomenon was previously demonstrated for quinine in proximal tubules (Escobar and Sitar, 1995). In isolated proximal tubules, the nonspecific rOCT1 and rOCT2 substrate choline and the rOCT2 > rOCT1-selective substrate guanidine weakly inhibited TEA uptake at higher concentrations and were without effect on amantadine uptake in KHS and CT, respectively (Fig. 10). The OCTN2 substrate carnitine did not inhibit amantadine or TEA uptake into proximal tubules in a dose-dependent manner in either KHS or CT (data not shown).

Discussion

By expressing rOCT1 and rOCT2 in *Xenopus* oocytes and HEK 293 cells, we were able to demonstrate that amantadine is translocated by both transporters. Also, amantadine uptake into rOCT1-transfected HEK 293 cells, but not rOCT2-transfected HEK 293 cells, was stimulated by bicarbonate. Its uptake by rOCT1 or rOCT2 is electrogenic, can be inhibited by quinine, cyanine$_{863}$, and procainamide, and exhibits similar inhibitor sensitivity, as previously demonstrated for TEA uptake (Arndt et al., 2001). The uptake of TEA in rat renal proximal tubules shows characteristics that are similar but not identical to the transport expressed by rOCT1 and rOCT2. Therefore, rOCT1 and rOCT2 might contribute to bicarbonate-dependent and -independent amantadine uptake in the proximal tubule. The data on amantadine uptake from proximal tubules compared with *Xenopus* oocytes and HEK 293 cells, however, strongly suggest that a transporter different from rOCT1 and rOCT2 is responsible for the bulk of bicarbonate-dependent amantadine uptake in the proximal tubule. Specifically, for several inhibitors, largely different potencies were observed for the inhibition of amantadine compared with TEA uptake in isolated proximal tubules or to inhibition of TEA uptake expressed by rOCT1 or rOCT2 in *Xenopus* oocytes. In isolated proximal tubules, the differences between the IC_{50} values for inhibition of TEA uptake and of bicarbonate-dependent amantadine uptake make it

TABLE 2

IC_{50} values (micromolar) for inhibition of [^{14}C]TEA (20 μM) or amantadine (10 μM) uptake into rOCT1 and rOCT2 expressing HEK 293 cells

Inhibition studies were performed in the presence (KHS) or absence (CT) of 25 mmol/l bicarbonate at pH 7.4. Transport assay duration was 3 s. IC_{50} values are reported as mean ± S.E.M. of three to six individual experiments. For inhibition of amantadine uptake (KHS buffer), the maximal concentration of cyanine$_{863}$ (200 μM) inhibited about 30 and 25% of rOCT1- and rOCT2-mediated amantadine uptake; thus, IC_{50} values were estimated to be greater than 200 μM. IC_{50} values were different.

Inhibitor	Substrate	rOCT1 HEK 293 Cells		rOCT2 HEK 293 Cells	
		KHS	CT	KHS	CT
Amantadine	TEA	500 ± 70	280 ± 40	210 ± 40*	290 ± 70
Procainamide	TEA	7.0 ± 1.0	12 ± 2	90 ± 16**	215 ± 5**†
Quinine	TEA	0.9 ± 0.1	1.1 ± 0.4	13 ± 4*	38 ± 7**†
Cyanine$_{863}$	TEA	0.4 ± 0.1	0.45 ± 0.07	1.0 ± 0.1	1.2 ± 0.2
	AM	>200 μM	89 ± 71***	>200 μM	15 ± 4***

AM, amantadine.
* $P < 0.05$, ** $P < 0.01$ compared to OCT1 within buffer group.
† $P < 0.05$ compared to rOCT2 in KHS.
*** $P < 0.001$ compared to cyanine$_{863}$ inhibition of TEA uptake (two-way ANOVA followed by Tukey's HSD test).

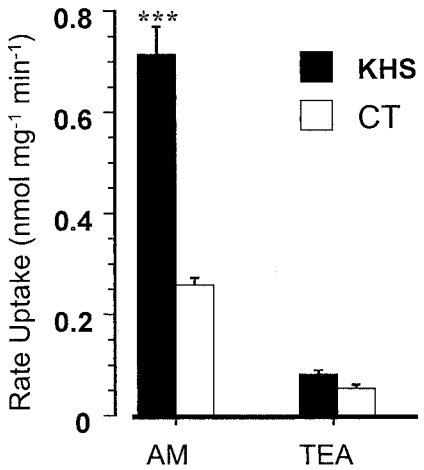

Fig. 8. Rate (nanomoles per milligram of protein per minute) of 10 μM [^{3}H]amantadine (AM) and 10 μM [^{14}C]TEA uptake into isolated proximal tubules in KHS or CT buffer at pH 7.4. Amantadine and TEA transport assays were 30 and 60 s, respectively. KHS buffer contained 25 mM bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). Each bar represents the mean ± S.E.M. of 6–11 separate determinations. ★★★, $P < 0.001$ compared with amantadine uptake in CT (ANOVA followed by Tukey's HSD test).

highly improbable that specific regulatory states of rOCT1 and/or rOCT2 in the proximal tubule are responsible for bicarbonate-dependent amantadine uptake. The observation that cyanine$_{863}$ inhibited amantadine uptake in *Xenopus* oocytes or HEK 293 cells more potently than in proximal tubules, but inhibited TEA uptake in HEK 293 cells with similar potency as in proximal tubules (Tables 1 and 2), also supports the hypothesis that proximal tubules contain a bicarbonate-dependent amantadine transporter in addition to rOCT1 and rOCT2.

Concerning the identity of this additional transporter, several cloned transporters deserve consideration. It could be the organic cation transporter 3 (rOCT3) that has a low affinity for TEA, guanidine, and procainamide and is transcribed in proximal and distal tubules (Kekuda et al., 1998; Wu et al., 2000). The observation that rOCT3 is potential-sensitive whereas amantadine uptake is independent of membrane potential argues against this possibility (Escobar and Sitar, 1995; Kekuda et al., 1998). The rat transporter Oatp1, which transports organic cations in addition to anions, can be ruled out because it has been localized to the brush-border membrane of proximal tubules and is inhibited by corticosterone (Bergwerk et al., 1996; Bossuyt et al., 1996; Kanai et al., 1996; van Montfoort et al., 2001), and it has a high affinity for digoxin (K_M 0.24 μM), which does not inhibit amantadine uptake into proximal tubules (Escobar and Sitar, 1996; Noe et al., 1997; Abe et al., 1998). Another candidate may be OCTN2, which mediates Na^+-independent transport of TEA and is inhibited by several other organic cations (Tamai et al., 1998; Wu et al., 1999). Nevertheless, OCTN2 cannot be the bicarbonate-dependent amantadine transporter because it is expressed in the luminal membrane of the proximal tubule (Ohashi et al., 2001; Tamai et al., 2001), and we observed that carnitine did not inhibit amantadine or TEA uptake into proximal tubules (data not shown). Recently, a verapamil transporter has been identified in human retinal epithelial pigment cells that has many similar properties to the bicarbonate-dependent amantadine transporter (Han et al., 2001). Both transporters are inhibited by quinidine but not by TEA or N^1-methylnicotinamide (Escobar et al., 1994; Escobar and Sitar, 1995; Goralski and Sitar, 1999; Han et al., 2001). Furthermore, verapamil displays bicarbonate-stimulated uptake and inhibits amantadine uptake into renal tubules (unpublished data). Future studies may clarify whether this verapamil transporter is identical to the bicarbonate-dependent amantadine transporter in the proximal tubule.

We confirmed that several inhibitors, including cyanine$_{863}$, quinine, and procainamide, have a greater potency against TEA transport by rOCT1 compared with rOCT2 (Arndt et al., 2001). We now demonstrated that amantadine also belongs to the inhibitors, which have a greater potency against TEA transport by rOCT2 compared with rOCT1, and discovered the unexpected observation that this selectivity is modulated by bicarbonate. In the presence of bicarbonate, the inhibitory potency of amantadine for rOCT1 was decreased, whereas that of procainamide and quinine for rOCT2 was increased. Future studies are necessary to elucidate whether these differential bicarbonate effects are due to 1) ion-pair formation of cationic ligands with bicarbonate, 2) bicarbonate binding to an allosteric site, or 3) antiport of organic cations influx with bicarbonate that may exhibit different kinetics and affinity to inhibitors than cation uniport.

TEA uptake data in the proximal tubule suggest the involvement of both rOCT1 and rOCT2. Involvement of rOCT1 is suggested since the IC_{50} values for inhibition of TEA uptake by quinine and procainamide in isolated tubules are similar to those for rOCT1 expressed in HEK 293 cells. In addition, neither in isolated proximal tubules nor after expression of rOCT1 in HEK 293 cells were the affinities of

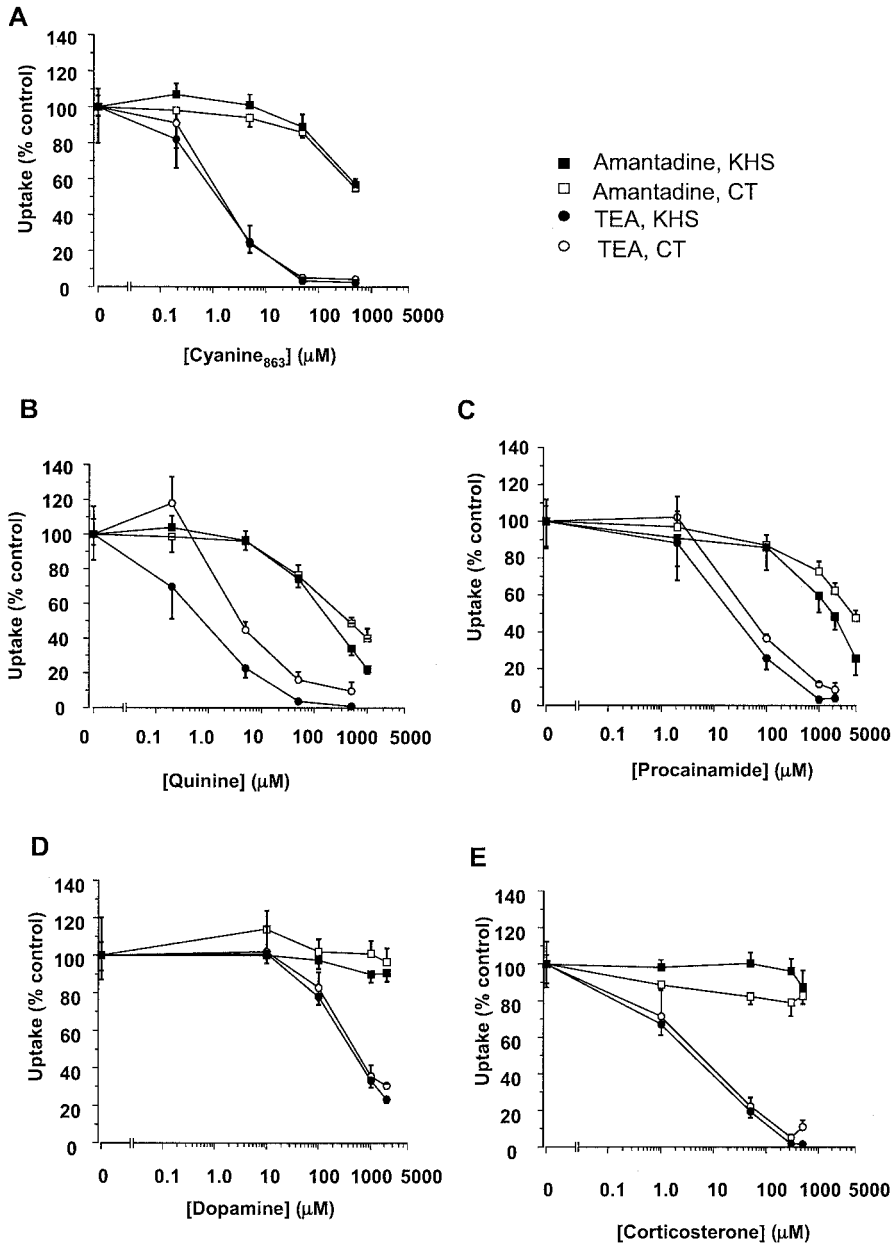

Fig. 9. Inhibition of 10 μM [^{3}H]amantadine and 10 μM [^{14}C]TEA transport into isolated proximal tubules in KHS or CT buffer at pH 7.4 by the OCT substrates or inhibitors cyanine$_{863}$ (A), quinine (B), procainamide (C), dopamine (D), and corticosterone (E). Amantadine and TEA transport assays were 30 and 60 s, respectively. KHS buffer contained 25 mM bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). Data are presented as uptake as a percentage of control amantadine or TEA uptake in the absence of inhibitors. Each symbol represents the mean ± S.E.M. of four to six separate determinations.

cyanine$_{863}$, quinine, and procainamide influenced by bicarbonate. Using 1 mM guanidine, only 40% inhibition of TEA uptake into proximal tubules was observed. Since the IC_{50} values of guanidine inhibition of TEA uptake mediated by rOCT1 and rOCT2 were 4.5 and 0.17 mM, respectively (Arndt et al., 2001), the fraction inhibited by 1 mM guanidine should be due to TEA transport by rOCT1. Involvement of rOCT2 in proximal tubular TEA uptake is suggested since the IC_{50} for corticosterone inhibition of its uptake was 3 to 5 μM. This observation is similar to the IC_{50} value of corticosterone for expressed rOCT2 (4 μM) and much lower than the IC_{50} value of 151 μM for rOCT1 (Arndt et al., 2001). These data, however, do not allow estimating the fractional contribution of both transporters in TEA uptake since the transport properties observed in the proximal tubule cannot be explained by a ratio of rOCT1 and rOCT2. This could mean that another unidentified transporter is involved. rOCT3 is unlikely to explain our observations because its K_M for TEA is 2.5 mM (Kekuda et al., 1998). Another possible explanation is that rOCT1 and rOCT2 exist in different regulatory states in the two systems. We showed that the affinity of TEA to rOCT1 was increased after phosphorylation of rOCT1 by protein kinase C, whereas the affinity of quinine was not changed (Mehrens et al., 2000). This interpretation is also supported by the observation that IC_{50} values for inhibition of TEA uptake into rOCT1- and rOCT2-expressing HEK 293 cells by amantadine were 4 to 5 times higher than those in the isolated proximal tubule (Goralski and Sitar, 1999). The finding that in proximal tubules only 40% of TEA uptake was blocked by 1 mM choline, a substrate for both rOCT1 and rOCT2 with a K_M between 0.3 and 0.6 mM (Koepsell, 1998; Arndt et al., 2001) could indicate the existence of an additional TEA transporter with a low affinity for choline or different regulatory states between rOCT1 and rOCT2 in proximal tubules compared with HEK 293 cells that affect affinity for choline.

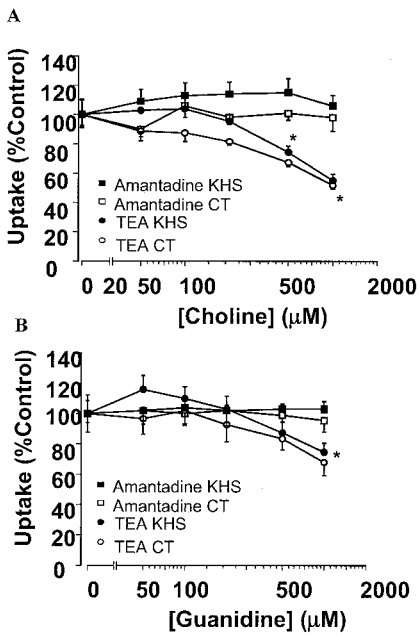

Fig. 10. Inhibition of 10 μM [^{3}H]amantadine and 10 μM [^{14}C]TEA transport into isolated proximal tubules in KHS and CT buffer by the OCT1 and OCT2 inhibitors choline (A) and guanidine (B). Amantadine and TEA transport assays were 30 and 60 s, respectively. KHS buffer contained 25 mM bicarbonate, and extracellular pH was adjusted to 7.4 by bubbling with O_2/CO_2 (95%/5%). Data are presented as uptake as a percentage of control amantadine or TEA uptake in the absence of inhibitors. Each symbol represents the mean ± S.E.M. of three to five separate determinations. ⋆, $P < 0.05$, TEA uptake in the presence of 500 or 1000 μM choline or 1000 μM guanidine was lower than respective control for assays in both KHS and CT buffer (two-way ANOVA followed by Tukey's HSD test).

rOCT1- and rOCT2-mediated organic cation transport was sensitive to modulation by bicarbonate. Bicarbonate differentially modulated the uptake of TEA and amantadine in the three transport systems used in our study. This observation supports the idea that bicarbonate modulation of organic cation transport by different cation transporters is linked to cell type-specific regulation. In HEK 293 cells, bicarbonate stimulated amantadine uptake only by rOCT1 and stimulated TEA uptake only by rOCT2. In *Xenopus* oocytes, bicarbonate inhibited TEA uptake by both rOCT1 and rOCT2 and did not affect amantadine uptake by these transporters. Thus, modulatory effects of bicarbonate on rOCT1 and rOCT2 transport function also depended on the transported substrate. The mechanism leading to this substrate-specific effect of bicarbonate remains to be determined. On the basis of mutagenesis studies showing that a conservative point mutation in rOCT1 increased the affinity for some substrates but not for others, we raised the hypothesis that polyspecific organic cation transporters contain a binding pocket with several interaction domains for structurally different cations (Gorboulev et al., 1999). One could imagine that bicarbonate binding at this pocket or at an allosteric site modulates the structure of this binding pocket, which may lead to cation-specific changes in affinity.

In summary, our findings support bicarbonate modulation of rOCT1 and rOCT2 function in HEK 293 cells and *Xenopus* oocytes. Nevertheless, these transporters do not mediate the major bicarbonate-dependent component of amantadine basolateral membrane uptake into the renal proximal tubule. The exact proportion of proximal tubule TEA uptake mediated by rOCT1 and rOCT2 cannot be determined in the present study because additional TEA transporters may exist and/or rOCT1 and rOCT2 may be in differential regulatory/functional states when expressed in cells versus isolated proximal tubules. It is clear from this study that demonstration of function of isolated expressed transporters may not always equate to function in freshly prepared renal tissue. Further molecular and in vivo characterizations of the OCTs [especially the amantadine transporter(s)] are required to more clearly identify their importance in drug elimination by the kidney.

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