Characterization of Guanidine Transport in Human Renal Brush Border Membranes

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Purpose. Organic cation transporters in the renal proximal tubule are important in the secretion of many clinically used drugs and their metabolites. The goal of this study was to determine the mechanisms of guanidine transport in human kidney.

Methods. Brush-border membrane vesicles were prepared from donor human kidneys deemed unsuitable for renal transplantation.

Results. Uptake of [^{14}C]-guanidine (50 μM) in the vesicles, as determined by rapid filtration, was significantly greater in the presence of an outwardly-directed proton gradient, at all early time points, than in the absence of the gradient. Proton-stimulated uptake of [^{14}C]-guanidine at 30 sec (32.0 ± 1.24 pmol/mg protein) was significantly inhibited by a number of organic cations including 5 mM unlabeled guanidine (14.8 ± 1.84 pmol/mg protein) and 5 mM MIBA (9.14 ± 3.80 pmol/mg protein), but not by 5 mM TEA (28.4 ± 5.67 pmol/mg protein). Guanidine, but not TEA, *trans*-stimulated [^{14}C]-guanidine uptake. Conversely, TEA, but not guanidine, *trans*-stimulated [^{14}C]-TEA uptake in the vesicles. The proton-dependent transport of guanidine was characterized by a K_m of 3.52 ± 0.42 mM (SE) and a V_{max} of 34.6 ± 8.64 pmol/mg protein/sec (SE).

Conclusions. These results demonstrate that guanidine transport in human renal brush border membrane vesicles is stimulated by a proton gradient. Evidence was obtained suggesting that the transporter for guanidine is distinct from the previously described organic cation proton antiporter for TEA.

KEY WORDS: brush-border membrane vesicles; organic cation transport; tetraethylammonium; guanidine; human kidney; transporters.

INTRODUCTION

Organic cation transporters in the renal proximal tubule are responsible for the secretion of many endogenous amines (e.g., choline, dopamine, and epinephrine) as well as a number of xenobiotics and drugs (e.g., cimetidine, morphine, and procainamide) (1–4). Studies using isolated renal cortical membrane vesicles suggest that organic cations are transported by distinct carrier-mediated processes in both brush-border and basolateral membranes (5–11). In the basolateral membrane, organic cations are transported largely via a facilitative, but passive, electrogenic carrier-mediated system (3,5,10–14). The driving force for this transporter is the cell's inside-negative membrane potential difference and/or an organic cation-organic cation exchange mechanism. Intracellular sequestration and concentrative transport into organelles may contribute to the steady-state accumulation of organic cations within the proximal tubule cell (8,15,16). Transport of organic cations into the lumen, across the brush-border membrane, occurs via a secondary active, organic cation-proton exchange mechanism (16–26). The lumen to cell inwardly-directed proton gradient, which serves as the driving force, is generated at the luminal membrane largely by the Na^+-H^+ antiporter and the H^+-ATPase responsible for bicarbonate reabsorption.

The luminal organic cation-proton exchange system, responsible for the transport of prototypical organic cations, tetraethylammonium (TEA) and N^1-methylnicotinamide (NMN), has been characterized in rat (26), rabbit (6,18,21,27) and human (22) renal brush-border membrane vesicles. This organic cation-proton exchange system has recently been designated OCPA1, for organic cation-proton antiporter 1 (28). In addition, Ganapathy and coworkers have characterized a second organic cation proton-antiporter, designated OCPA2, in rabbit renal, intestinal, and human placental brush-border membrane vesicles, which differs from OCPA1 in terms of its substrate selectivity (27–30). OCPA2 is selective for the endogenous organic cation, guanidine (i.e., guanidinium, pKa 12.5), interacts with 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA), but generally excludes TEA and NMN.

Previous studies from this laboratory have elucidated the characteristics of NMN and TEA transport in renal brush-border membrane vesicles (22), however it is not known whether guanidine is transported by similar mechanisms. The purpose of this investigation was to determine the mechanisms of guanidine transport in human renal brush-border membrane vesicles. The data demonstrate that guanidine transport in human renal brush border membrane vesicles is saturable, stimulated by a proton gradient and inhibited by a number of organic cations, but not by TEA or NMN. These data suggest that the transport mechanism for guanidine is distinct from the mechanisms involved in TEA and NMN transport in the human renal brush border membrane.

METHODS

Kidney Tissue

Human kidneys that were unsuitable for transplant were obtained from the Organ/Tissue Transplant Services at the University of California, San Francisco. All human kidneys were perfused per transplant protocols until delivered on ice to the laboratory. On arrival, the capsid and surrounding tissue were removed. The cortex was separated from the medulla by dissection and weighed. For those studies making use of fresh renal cortex, the brush-border membrane vesicles (BBMV) were prepared immediately. The remainder of the cortex was frozen in liquid nitrogen in 15- to 20-g portions and stored at −80°C until use.

Preparation of BBMV

BBMV were prepared by divalent cation precipitation and differential centrifugation as modified in our laboratory (21,31).

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ABBREVIATIONS: TEA, tetraethylammonium; NMN, N^1-methylnicotinamide; BBMV, brush border membrane vesicle.

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Briefly, for each study approximately 15–20 g of human cortex were minced with scissors. The cortex was homogenized in ice-cold buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5 mM ethylene glycol-bis(b-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 150 mM KCl, with pH adjusted to 7.4 with KOH. Magnesium sulfate (16 mM) was added to the homogenate which was stirred on ice for 20 min. Purified brush border membranes were obtained by a series of differential centrifugations, with the final pellet suspended in 10 mM HEPES and 150 mM KCl, pH 8.0 (HK buffer). For those studies in which the intravesicular pH was lowered, the BBMV were incubated with a 40- to 80-fold dilution of 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) and 150 mM KCl, pH 6.0 (MK buffer), at room temperature for 1 hour. The pellet was collected by centrifugation and resuspended. Previous studies of guanidine transport in rabbit kidney, intestine and human placenta brush border membrane visicles have shown that inorganic monovalent cations including potassium inhibited guanidine uptake (27,29,30). In preliminary studies we observed inhibitable guanidine transport in buffers containing potassium. Since potassium is known to reduce the nonspecific binding of organic cations to renal plasma membrane vesicles (26), we used potassium containing buffers in the preparation of the vesicles and in all of the studies described in this manuscript.

The protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as the standard. Enrichment of BBMV in the final preparation was determined by monitoring the enhancement of Na^+-K^+ ATPase, an enzyme marker for the basolateral membrane and alkaline phosphatase, an enzyme marker for the brush border membrane, activities in the pellet and the homogenate (22). The activities of these enzymes in the BBMV were enriched compared with the corresponding homogenates by the following factors: 9.2 to 14 for alkaline phosphatase and approximately 1.8 for Na^+-K^+ ATPase. All BBMV were kept on ice and used within 24 hours of preparation.

Uptake Studies

Studies were carried out using [^{14}C]-guanidine and [^{14}C]-TEA as model compounds. For the studies of organic cation transport in the absence of an outwardly directed proton gradient, 10 μl of BBMV in HK buffer were diluted with 40 μl of HK buffer containing approximately 50 μM [^{14}C]-guanidine or [^{14}C]-TEA and incubated for the various time periods as described in the figure legends. For the studies involving an outwardly directed proton gradient, 10 μl of BBMV in MK buffer were diluted with 40 μl of HK buffer containing 50 μM [^{14}C]-guanidine or [^{14}C]-TEA. For the studies of *trans*-stimulation, 10 μl of BBMV (preincubated with either 5 mM TEA or guanidine for 90 min at room temperature) were diluted with 90 μl of HK buffer containing 50 μM [^{14}C]-guanidine or [^{14}C]-TEA. At the end of the incubation time, the uptake was stopped by dilution with 3 ml ice-cold HK buffer and immediately filtered under vacuum (Hoefer Scientific Instruments, San Francisco, CA) through a membrane filter (0.3 μm, PH type, Millipore, Bedford, MA). The filter was then washed three times with 3 ml ice-cold buffer and placed into scintillant (Cytoscint-ES, ICN, Costa Mesa, CA). The radioactivity associated with the filter was determined by liquid scintillation counting (LS-1801, Beckman Instruments, Fullerton, CA). Nonspecific binding of 50 μM [^{14}C]-guanidine or [^{14}C]-TEA to the filter was subtracted from the total radioactivity associated with the filters. All transport studies were conducted at room temperature. Additional experimental details for individual studies are included in the legends.

Data Analysis

To obtain kinetic parameters for the transport of guanidine in human renal BBMV, the data were fit to a Michaelis-Menten model that included a linear, nonsaturable component such that rate = $[(V_{max} \cdot C)/(K_m + C) + K_{ns} \cdot C]$ where V_{max} is the maximal transport rate, K_m is the concentration needed to reach half of V_{max}, K_{ns} is the coefficient for the linear nonsaturable component, and C is the concentration of guanidine in the extravesicular solution. K_{ns} was defined as the rate constant of transport of guanidine in the presence of 5 mM MIBA, an amiloride analog which produces maximum inhibition of saturable guanidine uptake (28). K_{ns} was determined to be 2.22 pmol/(mg protein·sec·mM).

Unless otherwise specified, all data points were determined in triplicate and one to three experiments were performed in separate membrane preparations. Values represent means ± SD of a representative experiment or the mean ± SE of three replicate experiments conducted in separate membrane preparations. Statistical differences were determined by analysis of variance and Student's *t*-test. Probability at the 0.05 level was considered significant.

Chemicals

[^{14}C]-guanidine (56 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA; [ethyl-1-^{14}C]-TEA (53 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. All other chemicals were purchased from Sigma, St. Louis, MO or Fisher Scintific, Pittsburgh, PA and were of the highest grade available.

RESULTS

Effect of a H^+ Concentration Gradient and a Membrane Potential on Guanidine Uptake

Guanidine uptake in human renal brush-border membrane vesicles was enhanced in the presence of an outwardly directed proton gradient ($[H^+]_i > [H^+]_o$) (Figure 1). The uptake of guanidine (50 μM) in the vesicles was significantly greater in the presence of a proton gradient, at all early time points, than the corresponding uptake obtained in the absence of a proton gradient.

We further investigated the role of a proton gradient on the uptake of guanidine by studying the effect of carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), a proton ionophore, on the proton gradient-dependent guanidine uptake (Figure 2). Addition of FCCP abolished the proton gradient—stimulated guanidine uptake at early time points. The effect of FCCP was not due to an alteration in membrane potential since addition of FCCP, under voltage-clamped conditions (in the presence of valinomycin and in the absence of a K^+ gradient), resulted in a similar decrease in guanidine uptake to that observed with FCCP alone (Figure 2). Furthermore, imposition

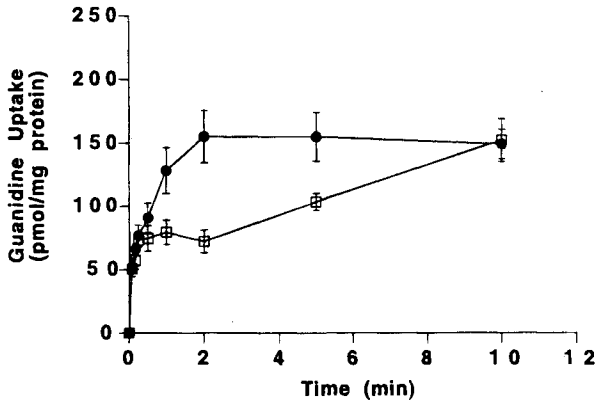

Fig. 1. Time-course of [^{14}C]-guanidine uptake in human renal BBMV in the presence (●) of an outwardly-directed proton gradient, where pH_{in}:pH_{out} = 6.0:8.0, and in the absence (□) of a proton gradient, where pH_{in}:pH_{out} = 8.0:8.0. In this experiment, vesicles were preloaded at room temperature for 1 hr with 10 mM MES and 150 mM KCl, pH 6.0 or 10 mM HEPES and 150 mM KCl, pH 8.0. The uptake buffer was 10 mM HEPES and 150 mM KCl, pH 8.0. The concentration of [^{14}C]-guanidine was 50 μM. Data are from one representative experiment (mean ± SD) of triplicate determinations.

of a large inside negative potential (created by an outwardly-directed K^+ gradient and valinomycin) did not affect inhibitable guanidine uptake (data not shown) suggesting that guanidine transport is not driven by membrane potential.

Kinetics of Guanidine Uptake

To determine the kinetic parameters of guanidine transport in human renal BBMV, we first identified a time at which accurate initial rates of transport could be determined. The proton-stimulated uptake of guanidine was linear for 30 s. Therefore, 30 s was used to determine the initial rate of guanidine transport. The initial rate of guanidine transport as a function of concentration is shown in Figure 3. The transport process was characterized by both a saturable and a nonsaturable, or linear, component. Figure 3 also shows the computer generated best-fit curve for the total transport process. For four separate experiments, the K_m was 3.52 ± 0.42 mM (SE) and the V_{max} was 34.6 ± 8.64 pmol/(mg protein·sec).

Effect of Organic Cations on Guanidine Uptake and TEA Uptake

The effect of TEA and various endogenous compounds on guanidine and TEA transport in renal brush-border membrane vesicles from the human kidney is shown in Table I. For inhibition studies, high concentrations (5 mM) were used of each test compound to determine if a compound had an effect on the transport of guanidine or TEA. Proton-stimulated guanidine uptake (30 s) was significantly inhibited by organic cations, but not by TEA. Conversely, proton-stimulated TEA uptake (30 s) was not inhibited by guanidine, but was inhibited by various organic cations. *p*-aminohippurate (PAH) did not inhibit the transport of either guanidine or TEA. Various clinically used drugs significantly inhibited guanidine and TEA transport (Figure 4a and 4b). Nicotine was the most potent inhibitor of guanidine transport and appeared to inhibit the diffusional component suggesting that at 5 mM nicotine may have non-specific membrane effects. The antiviral analogs, AZT and acyclovir, were weaker inhibitors of guanidine transport in comparison to the other drugs tested. Similar results were obtained for TEA transport except that AZT was a more potent inhibitor of TEA transport than was acyclovir. Foscarnet did not significantly inhibit TEA transport and was a weak inhibitor of guanidine transport.

Trans-Stimulation of Guanidine and TEA Uptake

To determine whether guanidine transport could be *trans*-stimulated by either guanidine or TEA, BBMV were pre-loaded with unlabeled guanidine or TEA and the uptake of radiolabeled

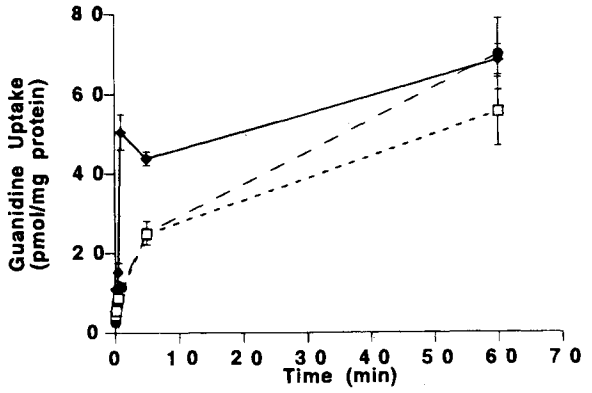

Fig. 2. Effect of a H^+-diffusion potential on [^{14}C]-guanidine uptake in human renal BBMV. In this experiment, vesicles were preloaded at room temperature for 1 hr with 10 mM MES and 150 mM KCl, pH 6.0. The uptake buffer was 10 mM HEPES and 150 mM KCl, pH 8.0. Uptake of 50 μM [^{14}C]-guanidine was measured in the presence of FCCP (●), FCCP and valinomycin with $[K^+]_{in}$ = $[K^+]_{out}$ (□), and in the absence of either FCCP or valinomycin (◆). Data are from a representative experiment (mean ± SD) of triplicate determinations.

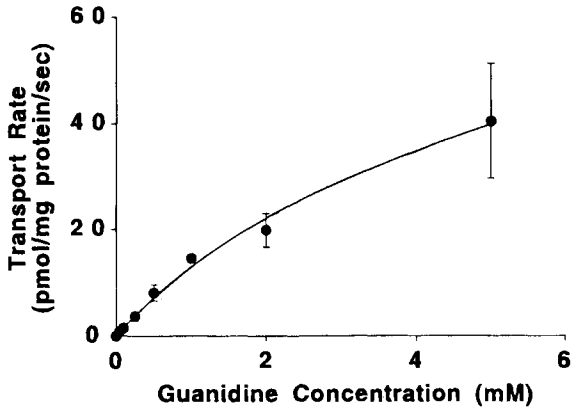

Fig. 3. Proton-stimulated inital rate of transport of [^{14}C]-guanidine (at 30 s) in human renal brush border membrane vesicles as a function of concentration of guanidine. In this experiment, vesicles were preloaded at room temperature for 1 hr with 10 mM MES and 150 mM KCl, pH 6.0. The uptake buffer was 10 mM HEPES and 150 mM KCl, pH 8.0 containing various concentrations of unlabeled guanidine. The concentration of [^{14}C]-guanidine was 50 μM. Data are expressed as mean ± SD of triplicate determinations.

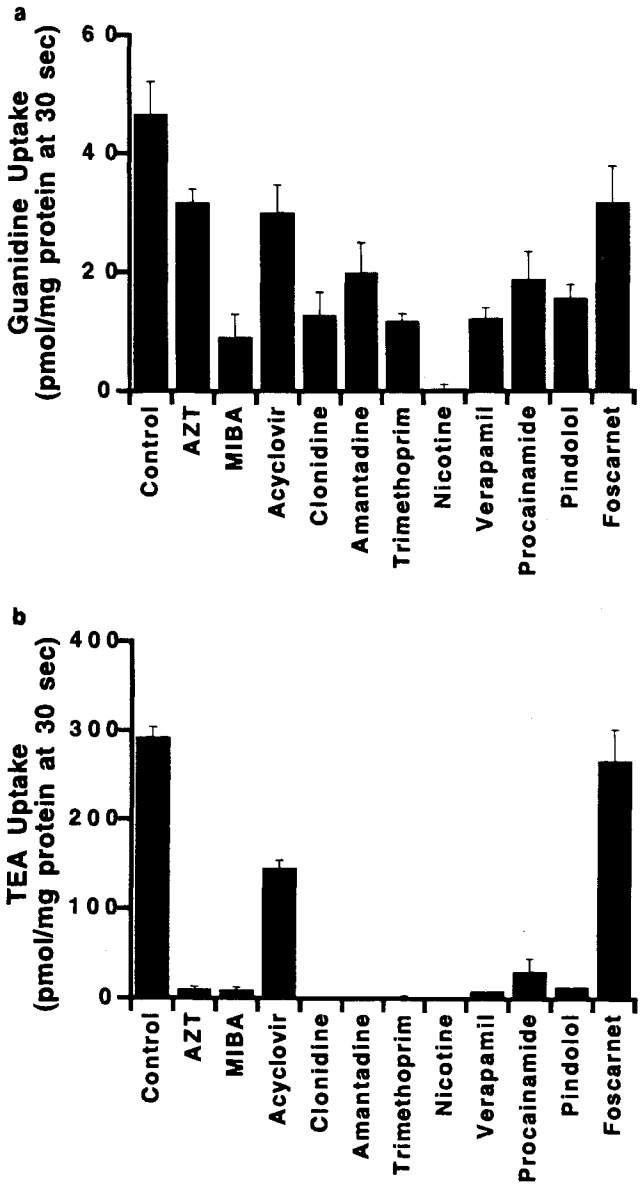

Fig. 4a and 4b. Uptake of 50 μM [^{14}C]-guanidine and 50 μM [^{14}C]-TEA in human renal BBMV (30 sec) with an outwardly-directed proton gradient (pH_{in}:pH_{out} = 6.0:8.0) in the absence (control) or presence of various inhibitors. In this experiment, vesicles were preloaded at room temperature for 1 hr with 10 mM MES and 150 mM KCl, pH 6.0. The uptake buffer was 10 mM HEPES and 150 mM KCl, pH 8.0. Inhibitor concentrations are at 5 mM except for trimethoprim (1 mM). Data are from one representative experiment (mean ± SD) of triplicate determinations. With the exception of Foscarnet, which did not significantly inhibit TEA uptake, all compounds significantly ($p < 0.05$) inhibited both TEA and guanidine uptake. The control uptake values for verapmil, procainamide, pindolol, and MIBA were 32.0 ± 1.24 and 307 ± 67.8 pmol/mg protein/30 sec for [^{14}C]-guanidine and [^{14}C]-TEA uptake, respectively.

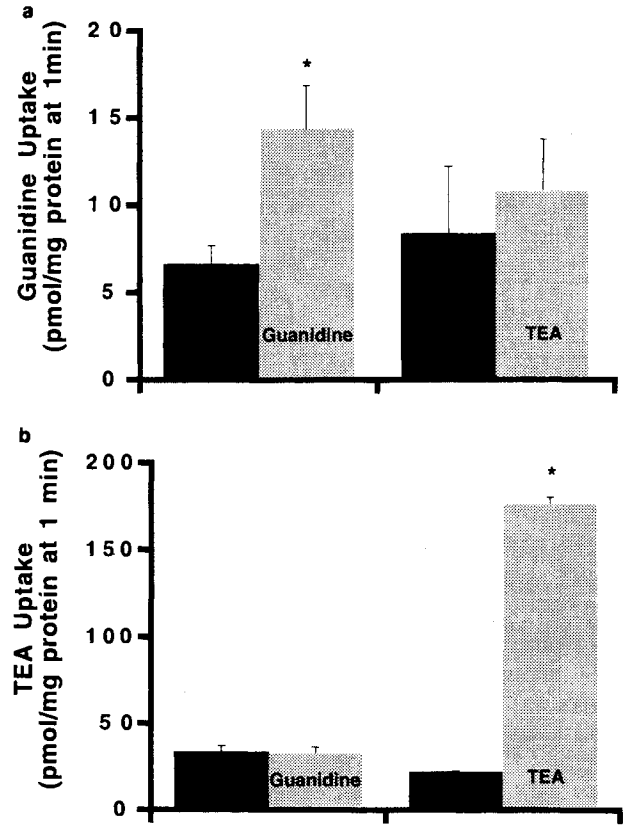

Fig. 5a and 5b. *Trans*-stimulation of [^{14}C]-guanidine (5a) and [^{14}C]-TEA uptake (5b). In this experiment, vesicles (light bars) were pre-incubated in 10 mM HEPES and 150 mM KCl, pH 8.0 buffer containing either TEA (5 mM) or guanidine (5 mM) at room temperature for 90 min. Control vesicles (dark bars) were incubated in 10 mM HEPES and 150 mM KCl, pH 8.0 buffer. The uptake of 50 μM [^{14}C]-guanidine or [^{14}C]-TEA was measured at 1 min. Data are from a representative experiment (mean ± SD) of triplicate determinations from a frozen kidney BBMV preparation. * $P < 0.05$.

guanidine or TEA was measured. In the absence of a proton gradient (pH_{in} = pH_{out} = 7.4), unlabeled guanidine (5 mM) *trans*-stimulated the uptake of [^{14}C]-guanidine (50 mM), whereas unlabeled TEA (5 mM) failed to produce a significant stimulatory effect (Figure 5a). In contrast, unlabeled guanidine (5 mM) did not *trans*-stimulate the uptake of [^{14}C]-TEA (50 mM), whereas unlabeled TEA (5 mM) significantly *trans*-stimulated [^{14}C]-TEA uptake (Figure 5b).

DISCUSSION

Early renal clearance studies established that tubular secretion, in addition to glomerular filtration, contributes greatly to the secretion and clearance of many organic cations. The classical model of organic cation secretion in the proximal tubule, namely, entry of organic cations across the basolateral membrane via a potential dependent organic cation transporter and efflux into the lumen via an organic cation-proton antiporter across the brush-border membrane (5,16), has been recently challenged. It now appears that several other functionally distinct transporters may be involved in the handling and transport of organic cations. These include the multidrug resistance (MDR) transporter, a choline transporter, and an ATP-stimulated TEA transporter (4,15,16,32–34). Furthermore, possible isoforms of the organic cation-proton antiporter (OCPA1 and OCPA2) have been postulated based on reported substrate specificities (28). OCPA1, which transports the prototypical organic cations, TEA and NMN, has been characterized in renal proximal tubules of dog (3,5,17,20,23), rat (26), rabbit

(1,6,12,21,25,27) and snake (19) and more recently in human kidney (22). OCPA2, which accepts guanidine and MIBA but excludes TEA and NMN as substrates, has been described in rabbit intestine and kidney and in human placenta (27–30). Interestingly, only OCPA2 is found in rabbit intestine and human placenta, whereas both OCPA1 and OCPA2 are found in rabbit renal brush border membranes (1,21,25,27).

In this study, evidence is presented for the presence of an organic cation transporter in human kidney brush-border membrane vesicles selective for the endogenous organic cation, guanidine. Consistent with the previous report in rabbit brush border membrane vesicles, the driving force for this transporter is an outwardly-directed proton gradient (Figure 1) and not an inside negative potential difference (Figure 2).

A saturable transport process consistent with a carrier-mediated transport system with a low affinity for guanidine (K_m = 3.5 mM) was observed. An Eadie-Hofstee plot of the data also suggested that there is only one saturable component (data not shown). This K_m is similar to the previously characterized low affinity transporter for guanidine in rabbit renal brush border membrane vesicles (27). Proton-stimulated guanidine uptake was inhibited by a number of organic cations, but not by the organic anion, PAH, and only weakly by Forscarnet, another organic anion, suggesting that the transporter is selective for organic cations.

Two lines of evidence suggest that the transporter for guanidine characterized in this study may be different from the previously characterized transporter for TEA and NMN in human renal brush-border membrane vesicles (22). First, TEA and NMN, even at high concentrations (5 mM) did not significantly inhibit proton-stimulated guanidine uptake (Table I). Conversely, guanidine (5 mM) did not inhibit proton-stimulated TEA uptake. Furthermore, although there were overlapping selectivities between the two transporters, quantitative differences in the magnitude of inhibition by various compounds were apparent (Table I, Figure 4).

Second, [^{14}C]-TEA uptake was *trans*-stimulated by unlabeled TEA, but not by guanidine, whereas [^{14}C]-guanidine uptake was *trans*-stimulated by unlabeled guanidine, but not by TEA (Figure 5). These data provide evidence that guanidine is transported into the intravesicular space by a transporter distinct from the transporter for TEA. However, the data do not exclude the possibility that the compounds are transported with large differences in their kinetic characteristics by the same mechanism.

The results of the present studies indicate that a number of clinically important drugs inhibit the transport of both guanidine and TEA suggesting that these two transporters have overlapping substrate selectivities. Many of these compounds including cimetidine, procainamide, pindolol, and clonidine, are actively secreted in the proximal tubule (9,16) and the data obtained in this study suggest the possibility that these transporters may play a role in their renal elimination. Further studies involving radiolabeled substrates or *trans*-stimulation studies will resolve if these compounds are translocated by carrier-mediated processes as suggested in our inhibition studies. The finding that the amiloride analog, MIBA, inhibits to a similar extent both proton gradient-driven guanidine and TEA uptake is consistent with previous results in rabbit kidney brush border membranes (27) and is of interest since amiloride analogs also bind to Na^+/H^+ antiporters. Because human kidney for research is scarce, it was not possible to conduct the mechanistic studies required to determine the mechanisms or potencies of individual compounds in inhibiting guanidine uptake.

The relative importance of each transporter in secreting a particular organic cation or drug under physiologic conditions will depend on the relative affinity of the transporter for the organic cation (or drug) as well as the V_{max}. V_{max} depends upon the turnover rate constant and the number of functional transporters in the membrane. The V_{max} of guanidine obtained in this study (34.6 pmol/mg protein/sec) is similar to that obtained previously for NMN in human renal brush border membrane vesicles (24.4 pmol/mg protein/sec) (22) suggesting that the product of the number of functional transporters and the turnover rate constants of the two transporters loaded with these substrates is similar.

Table I. Effect of Inhibitors on [^{14}C]-TEA and [^{14}C]-Guanidine Uptake in Human Renal Brush Border Membrane Vesicles

	[^{14}C]-Guanidine uptake (pmol/mg protein/30 sec)	Control (%)	[^{14}C]-TEA uptake (pmol/mg protein/30 sec)	Control (%)
Control	32.0 ± 1.2	100	307 ± 69.8	100
Guanidine	14.8 ± 1.8[a]	46.2	335 ± 35.9	109
TEA	28.4 ± 5.7	88.8	24.5 ± 1.6[a]	8.0
Choline	21.9 ± 1.0[a]	68.4	156 ± 4.6[a]	50.9
Arginine	17.0 ± 2.5[a]	53.1	319 ± 20.0	104
Creatinine[b]	17.9 ± 3.27[a]	38.4	103 ± 8.42[a]	35.3
NMN[c]	34.3 ± 3.99	94.8	—	—
PAH	28.0 ± 8.7	87.5	318 ± 11.9	104

Note: The uptake of [^{14}C]-TEA (50 μM) and [^{14}C]-guanidine (50 μM), at 30 s, in renal brush border membrane vesicles from human kidney in the presence of an outwardly-directed proton gradient (pH_{in}:pH_{out} = 6.0:8.0) in the absence (control) and presence of various inhibitors. In this experiment, vesicles were preloaded at room temperature for 1 hr with 10 mM morpholinoethanesulfonic acid (MES) and 150 mM KCl, pH 6.0. The uptake buffer was 10 mM HEPES and 150 mM KCl, pH 8.0. Each inhibitor was present at 5 mM. Data are from one representative experiment (mean ± SD) of triplicate determinations from a frozen kidney BBMV preparation.

[a] Significantly different from control, ($p < 0.05$).

[b] In the studies of creatinine inhibition, the control for the guanidine experiment was 46.6 ± 5.50 pmol/mg protein and for the TEA experiment was 292 ± 11.3 pmol/mg protein/30 sec.

[c] In the studies of NMN inhibition, the control for the guanidine experiment was 36.2 ± 6.26 pmol/mg protein/30 sec.

In summary, this study demonstrates that guanidine transport in human renal brush border membrane vesicles is enhanced by a proton gradient and inhibited by a number of organic cations, but not by TEA or NMN. The data suggest that the transport mechanisms for guanidine may be distinct from the mechanisms involved in TEA and NMN transport in the human renal brush border membrane.

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