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Selective Substrates for Non-Neuronal Monoamine Transporters

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ABSTRACT

The recently identified transport proteins organic cation transporter 1 (OCT1), OCT2, and extraneuronal monoamine transporter (EMT) accept dopamine, noradrenaline, adrenaline, and 5-hydroxytryptamine as substrates and hence qualify as non-neuronal monoamine transporters. In the present study, selective transport substrates were identified that allow, by analogy to receptor agonists, functional discrimination of these transporters. To contrast efficiency of solute transport, stably transfected 293 cell lines, each expressing a single transporter, were examined side by side in uptake experiments with radiolabeled substrates. Normalized uptake rates indicate that tetraethylammonium, with a rate of about 0.5 relative to 1-methyl-4-phenylpyridinium (MPP^+), is a good substrate for OCT1 and OCT2. It was not, however, accepted as substrate by EMT. Choline was transported exclusively by OCT1, with a rate of about 0.5 relative to MPP^+. Histamine was a good substrate with a rate of about 0.6 relative to MPP^+ for OCT2 and EMT, but was not transported by OCT1. Guanidine was an excellent substrate for OCT2, with a rate as high as that of MPP^+. Transport of guanidine by OCT1 was low, and transport by EMT was negligible. With the guanidine derivatives cimetidine and creatinine, a pattern strikingly similar to guanidine was observed. Collectively, these substrates reveal key differences in solute recognition and turnover and thus challenge the concept of "polyspecific" organic cation transporters. In addition, our data, when compared with previous studies, suggest that OCT2 corresponds to the organic cation/H^+ antiport mechanism in renal brush-border membrane vesicles, and that EMT corresponds to the guanidine/H^+ antiport mechanism in membrane vesicles from placenta and intestine.

The physiological actions of the majority of released neurotransmitters are terminated by integral plasma membrane transport proteins that actively remove the transmitters from extracellular space. These transporters thus profoundly control chemical signal transduction. Monoamine transmitters such as dopamine, noradrenaline, adrenaline, and 5-hydroxytryptamine are inactivated by two distinct transport mechanisms, which, by reference to localization, have been termed neuronal and non-neuronal (extraneuronal). Transporters of the neuronal type depend on Na^+ and Cl^- and show high affinity for monoamines. They are predominantly expressed in nerve endings, but are also found in certain non-neuronal cells. In the past, neuronal transporters have been cloned and intensively studied. Among these, the dopamine transporter DAT is a prominent example for its sensitivity to cocaine (Povlock and Amara, 1997). Non-neuronal monoamine uptake, on the other hand, has been described as a Na^+-independent, high-capacity transport mechanism.

Recently, three proteins have been cloned that accept catecholamines and 5-hydroxytryptamine as substrates, and hence qualify as non-neuronal monoamine transporters. They are OCT1 (Gründemann et al., 1994; Nagel et al., 1997; Breidert et al., 1998), OCT2 (Okuda et al., 1996; Gründemann et al., 1997, 1998b; Gorboulev et al., 1997; Busch et al., 1998), and EMT (Gründemann et al., 1998a; Kekuda et al., 1998), all members of the amphiphilic solute facilitator family of transport proteins (Schömig et al., 1998). Like the neuronal noradrenaline and dopamine transporters and the vesicular monoamine transporters, all three efficiently transport 1-methyl-4-phenylpyridinium (MPP^+). In fact, MPP^+ is the best substrate reported so far for each of OCT1, OCT2, and EMT. Even though OCT1 is confined to liver, kidney, and intestine, and OCT2 has been detected solely in kidney and brain, EMT has a broad tissue distribution. OCT1 and OCT2 are expected to move organic cations, e.g., metabolites and drugs, across cell membranes and thus take part in elimination of these compounds. OCT2 may be involved in renal handling of dopamine (Gründemann et al., 1998b). In vivo evidence implies that EMT contributes strongly to the inactivation of circulating catecholamines (Eisenhofer et al., 1996). Because all three are sensitive to many inhibitors, they have been designated as "polyspecific" (Gorboulev et al., 1997; Kekuda et al., 1998) or "multispecific" (Urakami et al.,

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ABBREVIATIONS: OCT, organic cation transporter; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; TEA, tetraethylammonium; MPP^+, 1-methyl-4-phenylpyridinium; EMT, extraneuronal monoamine transporter.

1998), vague terms imply that diffuse or multiple modes of substrate recognition. It must be emphasized, however, that it is not possible to infer transport efficiency from inhibition experiments. Notably, no clear differences in substrate specificity have been reported.

Based on pairwise comparisons of the human amino acid sequences, OCT1 and OCT2 are markedly homologous (70% identity, 84% similarity). EMT shows larger evolutionary distances to both OCT1 (50%, 70%) and OCT2 (50%, 73%). Although these numbers suggest that EMT is equally similar in structure to OCT1 and OCT2, previous pharmacological characterizations have revealed that EMT closely resembles OCT2, but not OCT1. In particular, disprocynium 24 and decynium 22, with K_i values around 10 nM the most potent inhibitors recognized so far, do not distinguish OCT2 and EMT (Gründemann et al., 1997, 1998a). The same holds for corticosterone, another potent competitive inhibitor, with K_i values around 200 nM. These inhibitors are far less effective with OCT1, which may thus be identified easily (Martel et al., 1996). At present, *O*-methylisoprenaline is the only compound that clearly discriminates EMT (K_i = 2 μM) from OCT2 (K_i = 600 μM).

This lack of selective high-affinity inhibitors and reservations against the concept of "polyspecificity" prompted us to search for substrates that would allow, in analogy to receptor agonists, functional discrimination of OCT1, OCT2, and EMT. Ideally, selective substrates would be efficiently transported by one transporter, but not by the others. Such substrates should then be useful not only as tools for in vitro and in vivo analysis of transporter function, but could also provide important clues to physiological function.

Materials and Methods

Assembly of Authentic Rat OCT2 (OCT2r). Total RNA was extracted by the method of Chomczynski and Sacchi (1987). Polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), and sequencing were performed as described (Gründemann et al., 1997).

An OCT2r cDNA with an authentic open reading frame was assembled as follows: a DNA fragment covering the critical purine stretch (see *Results*) was generated by RT-PCR with RNA from rat substantia nigra with forward primer 5′-CCG CTA TCC CTG GGC TGT GTC AAA and reverse primer 5′-TGG CCC ACA GCT CCC TTG GGT ATT. The expected amplicon (795 base) was isolated by UV-protected agarose gel electrophoresis (Gründemann and Schömig, 1996) and cloned into the *Sma*I site of pUC19 as described (Schömig et al., 1998). Sequencing revealed an A_6 stretch instead of A_7. The A_6 stretch was isolated, contained in a 294-base fragment, from the RT-PCR fragment by restriction with *Apo*I and *Nco*I. It was used to replace the corresponding fragment in pBlueOCT2r, which is composed of the artificial cDNA of OCT2r in pBluescript II SK (−) (Stratagene, Amsterdam, the Netherlands; Gründemann et al., 1997). The correction was verified by sequencing. Finally, the authentic OCT2r cDNA[1] was inserted into the *Xho*I and *Hin*dIII sites of pcDNA3 (Invitrogen, NV Leek, the Netherlands) to produce pcDNA3OCT2r.

Control of *Taq* DNA Polymerase. To test whether *Taq* DNA polymerase would accurately amplify the above mentioned A_7 stretch of OCT2r, at first a cRNA was generated by in vitro transcription. *Xho*I-linearized pcDNA3OCT2r (0.4 μg, containing the A_7 stretch) was incubated for 2 h at 37°C in 40 mM triethanolamine-HCl, pH 7.5 (37°C), 1.8 mM each of ATP, CTP, GTP, and UTP, 13.2 mM $MgCl_2$, 5 mM dithiothreitol, 5 U/ml inorganic pyrophosphatase from yeast, 0.5 U/μl RNase inhibitor from human placenta, and 50 U T7 RNA polymerase in a final volume of 25 μl. After incubation with 2 U RQ1-DNase (Promega, Mannheim, Germany) for 30 min at 37°C, RNA was purified by extraction with phenol and chloroform and precipitated with ethanol. Of this cRNA, about 0.5 μg, as judged by gel electrophoresis, was reverse transcribed with an oligo(dT) primer-linker as described (Gründemann et al., 1997). In a parallel negative control, reverse transcriptase was omitted. After RNase A treatment, PCR was performed with forward primer 5′-CCG GGT CTG GGA CCT TCG and reverse primer 5′-GCG GAC GGT CTG CTT GCT TGA CTT GGA GGT AAA TTC TCT T (annealing temperature 55°C). The expected amplicon of 1437 bases, which was absent in the negative control, was cut with *Apo*I to produce a fragment of 946 bases. This was inserted into *Eco*RI-linearized pOCUS-2 (Novagen, Madison, WI). After electroporation of *Escherichia coli* DH10B, four clones were sequenced.

Cell Culture and Transfection. 293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were grown at 37°C in a humidified atmosphere (5% CO_2) on plastic culture flasks (Falcon 3112, Becton Dickinson, Heidelberg, Germany). The medium was Dulbecco's modified Eagle's medium (cat. no. 31885–023, Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (Life Technologies). Medium was changed every 2 to 3 days and the culture was split every 7 days.

293 cells were transfected with supercoiled plasmid DNA by lipofection with the Tfx-50 reagent according to the protocol of the vendor (Promega). Stably transfected cells were then selected with G418 (Life Technologies) as described (Gründemann et al., 1997). Expression of OCT2r was verified by RT-PCR and functional characterization.

Transport Assays. For measurement of uptake of radiolabeled solutes, cells were grown in surface culture on 60-mm polystyrol dishes (Nunclon 150288, Nunc, Wiesbaden, Germany) precoated with 0.1 g/l poly-L-ornithine in 0.15 M boric acid-NaOH, pH 8.4. After 2 to 3 days in culture (70–95% confluence), the cells were used for uptake experiments.

Uptake was measured at 37°C. After a preincubation period of 20 min with buffer A (125 mM NaCl, 25 mM HEPES-NaOH, pH 7.4, 5.6 mM (+)-glucose, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$), the cells were incubated with 100 nM ^{3}H- or ^{14}C-labeled substrates in buffer A. Incubation was stopped by rinsing the cells four times with 4 ml of ice-cold buffer A. Subsequently, the cells were solubilized with 0.1% (v/v) Triton X-100 in 5 mM Tris-HCl, pH 7.4, and radioactivity was determined by liquid scintillation counting. (+)-Ascorbic acid (1 mM) was present in experiments with dopamine to prevent oxidation.

Generally, inhibitors of transport were present during both the preincubation and uptake periods. This is necessary to achieve equilibrium binding with effective inhibitors, which are used at very low concentrations, and hence realize their full potency. However, because we had observed *trans*-stimulation effects with guanidine as inhibitor of OCT2r-mediated MPP^+ uptake, all inhibitors with a K_i larger than 1 μM were absent during preincubation. By contrast, potent inhibitors (K_i smaller than 1 μM) are at best transported very slowly and thus do not cause *trans*-stimulation.

Protein Determination. Protein was measured by a modification of the Bradford method (Zor and Selinger, 1996) with bovine serum albumin as standard.

Calculations and Statistics. Analysis of the time course of substrate accumulation was based on a one-compartment model as described earlier (Russ et al., 1992). Saturation curves were analyzed as described (Schömig et al., 1993). For the calculation of IC_{50} values, the Hill equation for multisite inhibition was fitted to the data by a nonlinear regression method. IC_{50} values are identical with K_i values, because nonsaturating substrate concentrations were

[1] The nucleotide sequence of authentic OCT2r has been submitted to the GenBank/European Bioinformatics Institute Data Bank with accession number Y13154.

used. K_m and K_i values are given as geometric mean with 95% confidence interval. Arithmetic means are given with S.E.M.

Amino acid sequences were analyzed with the Wisconsin Package as implemented in Heidelberg Unix Sequence Analysis Resources (HUSAR) [Deutsches Krebsforschungszentrum (German Cancer Research Center), Heidelberg, Germany]. Pairwise sequence alignments were computed with default parameters with the program GAP, part of the Wisconsin Package.

Drugs. The following radiolabeled drugs were used: MPP^+ iodide [H-3, 2200 Bq/pmol; ART-150, American Radiolabeled Chemicals Inc. (ARC), St. Louis, MO), dihydroxyphenylethylamine (dopamine; H-3, 1500 Bq/pmol; NET-131, New England Nuclear, Boston, MA], tetraethylammonium bromide (C-14, 2.0 Bq/pmol; ARC-577, ARC), choline chloride (H-3, 3200 Bq/pmol; TRK593, Amersham Pharmacia, Freiburg, Germany), histamine dihydrochloride (H-3, 1900 Bq/pmol; TRK631, Amersham Pharmacia), guanidine (C-14, 2.0 Bq/pmol; ARC-350, ARC), cimetidine (H-3, 460 Bq/pmol; TRK615, Amersham Pharmacia), and creatinine (C-14, 2.1 Bq/pmol; ARC-177, ARC).

The following drugs were used as substrates or inhibitors: MPP^+ iodide (D-048, Research Biochemicals, Inc., Natick, MA), 3-hydroxytyramine hydrochloride (56610, Fluka, Buchs, Switzerland), choline chloride (C7970–0, Aldrich, Steinheim, Germany), histamine dihydrochloride (4370, Merck, Darmstadt, Germany), guanidine hydrochloride (G-3272, Sigma, Deisenhofen, Germany), cimetidine (28541–2, Aldrich), and creatinine (27910, Fluka). Disprocynium24 was synthesized as described previously (Russ et al., 1993).

All other chemicals were of analytical grade.

Results

Authentic Primary Structure of OCT2r. According to the amino acid sequences stored in the public databases [GenBank/European Bioinformatics Institute Data Bank accession numbers D83044 (Okuda et al., 1996) and X98334 (Gorboulev et al., 1997)], OCT2r has an unusual C terminus that is 38 amino acids longer than those of the highly homologous transporters pig OCT2, human OCT2, and mouse OCT2. Underlying the beginning of deviation is a peculiar region of the OCT2r mRNA, with seven consecutive adenines contained in a stretch of 24 bases entirely made of purines.

By RT-PCR we cloned OCT2r fragments that were found to contain only six adenines. For control, we synthesized by in vitro transcription a cRNA with seven consecutive adenines in the purine stretch (see *Materials and Methods* for details). This cRNA was reverse transcribed, amplified with *Taq* DNA polymerase, inserted into a plasmid vector, and cloned into *E. coli* by standard procedures. Of four clones that were sequenced, one carried a A → G substitution and a deletion (Fig. 1a, clone #2), which indicates that the purine stretch is a difficult template for DNA polymerases. With the other three clones, however, the original sequence was recovered. Thus, we conclude that the A_6 stretch in the OCT2r fragment from RT-PCR does not represent a PCR artifact. Conversely, in the course of standard, PCR-independent cloning of OCT2r, there probably is a high chance of converting the A_6 stretch to A_7. Six adenines will translate into a C terminus that is identical in length and highly similar in amino acid sequence to its orthologs (Fig. 1b). The extra adenine, on the other hand, causes a frame shift, which leads to a much longer, artificial C terminus. Thus, we propose as authentic a OCT2r mRNA with six instead of seven consecutive adenines in the critical purine stretch. A similar cDNA synthesis artifact has been observed with cardiac titin cDNA (S. Labeit, personal communication).

Functional Expression of Authentic OCT2r. For functional expression, an authentic cDNA of OCT2r was assembled and inserted into the eucaryotic expression vector pcDNA3. The resulting plasmid, pcDNA3OCT2r, was used to stably transfect 293 cells, a transformed cell line derived from human embryonic kidney. Nonspecific and endogenous uptake was measured with control cells that were transfected with vector DNA.

Functional expression of OCT2r was verified in uptake experiments with cells in surface culture on plastic dishes. A detailed analysis of the time course of uptake of $[^3H]MPP^+$ into pcDNA3OCT2r-transfected cells (Fig. 2) revealed rate constants for inwardly (k_{in}) and outwardly (k_{out}) directed MPP^+ fluxes of 90 ± 9 μl min^{-1} mg $protein^{-1}$ and 0.9 ± 0.1 min^{-1}, respectively (n = 12). The uptake at equilibrium (A_{max}) amounted to 10.0 ± 0.2 pmol mg $protein^{-1}$. Based on an intracellular water space of 6.7 μl mg $protein^{-1}$ (Martel et al., 1996) and a transfection efficiency of 100%, at equilibrium a 15-fold accumulation of MPP^+ relative to medium can be estimated. MPP^+ uptake was completely inhibited by 10 μM disprocynium 24. An uptake period of 1 min was chosen for subsequent experiments to approximate initial rates of transport.

Expressed uptake, i.e., total uptake minus total uptake into control cells, of MPP^+ was saturable (Fig. 3a), with an apparent Michaelis-Menten constant, K_m, of 9.4 (95% confidence interval, 8.4–10.5) μM and a maximal uptake rate, V_{max}, of 0.47 ± 0.01 nmol min^{-1} mg $protein^{-1}$.

Furthermore, expressed uptake of ^3H-dopamine was saturable (Fig. 3b), with an apparent K_m of 2.3 (1.4–3.9) mM and a V_{max} of 4.4 ± 0.5 nmol min^{-1} mg $protein^{-1}$. For both MPP^+ and dopamine, the Eadie-Hofstee plot is compatible with a single uptake mechanism.

Comparison of Transport Substrates. To contrast efficiency of solute transport, stably transfected 293 cell lines expressing EMTh (Gründemann et al., 1998a), OCT2r (see above), or OCT1r (Breidert et al., 1998) were examined side

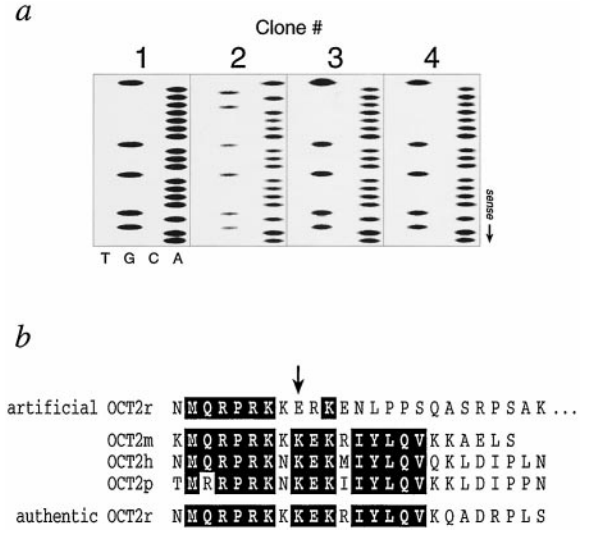

Fig. 1. Authentic primary structure of OCT2r. a, part of a sequencing gel that shows the critical purine stretch of OCT2r for four clones that were generated from a cRNA with an A_7 stretch by RT-PCR and standard plasmid cloning into *E. coli*. b, amino acid sequence alignment of artificial and authentic OCT2r with its orthologs from mouse (m), human (h), and pig (p). The arrow indicates start of frame shift in artificial OCT2r.

by side in uptake experiments with radiolabeled substrates. Control cells had been stably transfected with vector pcDNA3 (see above). Figure 4 (top) shows total uptake by the four cell lines of tetraethylammonium (TEA), choline, histamine, or guanidine at a concentration of 0.1 μM. To correct for uptake by control cells and for transporter number, the expressed uptake was divided by the expressed uptake of MPP^+, which was measured in paired assays (Fig. 4, bottom. See *Discussion* for rationale). These data allow direct comparison of transport efficiency for a particular solute by EMTh, OCT2r, or OCT1r.

With a factor of about 0.5 relative to MPP^+ uptake, TEA is a good substrate for OCT1r and OCT2r. TEA is not, however, accepted as substrate by EMTh. In the measurement of choline uptake, an endogenous uptake activity in 293 cells created a high background. Nevertheless, it is apparent that only OCT1r transports choline. The corrected uptake suggests choline to be as good a substrate as TEA for OCT1r. Choline uptake via OCT2r or EMTh was not significantly different from control. Histamine is a good substrate, with a factor of about 0.6 relative to MPP^+ uptake, for OCT2r and EMTh, but is poorly transported by OCT1r. Finally, guanidine is an excellent substrate for OCT2r, with an uptake comparable to that of MPP^+. Transport of guanidine by OCT1r is relatively low, and transport by EMTh is negligible.

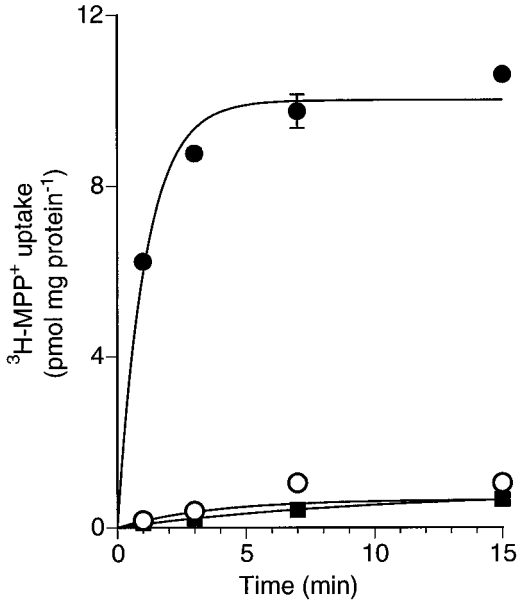

Fig. 2. Time course of MPP^+ uptake into 293 cells expressing authentic OCT2r. Cells grown in dishes were incubated at 37°C with 0.1 μM MPP^+. Shown is mean ± S.E.M. (n = 3). Exponential functions were fitted to the experimental data (Schömig et al., 1992) for control cells (stably transfected with vector alone; ○: k_{in} = 1.8 ± 0.1 μl min^{-1} mg protein^{-1}, k_{out} = 0.28 ± 0.03 min^{-1}) and OCT2r cells in the presence of 10 μM disprocynium 24 (also during preincubation; ■: k_{in} = 0.83 ± 0.05 μl min^{-1} mg protein^{-1}, k_{out} = 0.10 ± 0.01 min^{-1}).

Transport and Recognition of Histamine. The uptake of histamine mediated by OCT2r and EMTh was analyzed in more detail. An analysis of the time course of uptake (Fig. 5a) revealed similar rate constants for histamine fluxes for OCT2r (k_{in} = 28 ± 3 μl min^{-1} mg protein^{-1}, k_{out} = 0.51 ± 0.06 min^{-1}, n = 12) and for EMTh (k_{in} = 24 ± 2 μl min^{-1} mg protein^{-1}, k_{out} = 0.30 ± 0.03 min^{-1}, n = 12) (control cells: k_{in} = 0.49 ± 0.07 μl min^{-1} mg protein^{-1}, k_{out} = 0.12 ± 0.03 min^{-1}, n = 12).

Expressed uptake of histamine was saturable (Fig. 6, a and b), with small differences between OCT2r (K_m = 540 (350–840) μM, V_{max} = 8.5 ± 0.6 nmol min^{-1} mg protein^{-1}) and EMTh (K_m = 180 (66–480) μM, V_{max} = 5.6 ± 0.7 nmol min^{-1} mg protein^{-1}).

Inhibition by histamine of MPP^+ uptake was quantitatively examined for OCT1r, OCT2r, and EMTh (Fig. 7a). Even though the K_i values for OCT2r [0.39 (0.28–0.54) mM] and EMTh [0.14 (0.13–0.15) mM] are similar and correspond well to the respective K_m values, the K_i for OCT1r [1.4 (1.3–1.6) mM] was 4 or 10 times higher, respectively. Thus, the affinity of OCT1r for histamine is moderately reduced compared with OCT2r and EMTh. This altered recognition explains only partly why OCT1r does not appreciably transport histamine.

Transport and Recognition of Guanidine. The uptake of guanidine mediated by OCT2r was analyzed in more detail. Analysis of the time course of uptake of guanidine into pcDNA3OCT2r-transfected cells (Fig. 5b) revealed the following rate constants: k_{in} = 51 ± 8 μl min^{-1} mg protein^{-1},

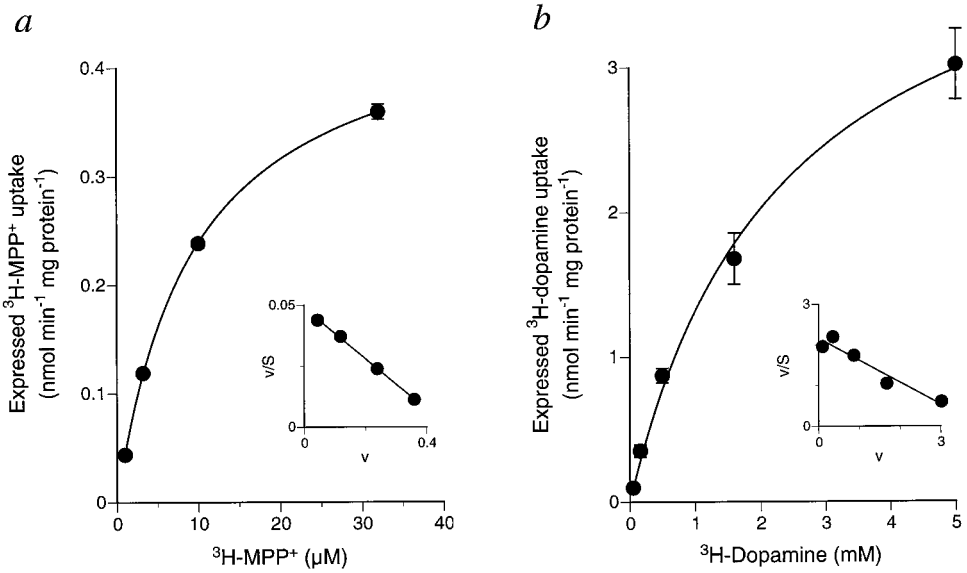

Fig. 3. Saturation of expressed uptake of MPP^+ (a) or dopamine (b) into 293 cells expressing authentic OCT2r. An uptake period of 1 min (MPP^+) or 1.75 min (dopamine) was chosen to approximate initial rates of transport. Shown is mean ± S.E.M. (n = 3). Expressed uptake was calculated by subtraction from total uptake of uptake into cells transfected with vector pcDNA3 alone. This control uptake increased linearly with substrate concentration, slope = 1.29 (MPP^+) or 1.08 μl min^{-1} mg protein^{-1} (dopamine). Insets: Eadie-Hofstee transformation.

k_{out} = 1.0 ± 0.2 min^{-1}, n = 12 (control cells: k_{in} = 1.48 ± 0.07 $\mu l\ min^{-1}\ mg\ protein^{-1}$, k_{out} = 0.049 ± 0.008 min^{-1}, n = 12).

Expressed uptake of guanidine was saturable (Fig. 6c), with K_m = 730 (520–1000) μM and V_{max} = 28 ± 1 nmol min^{-1} mg $protein^{-1}$.

Inhibition by guanidine of MPP^+ uptake was quantitatively examined for OCT1r, OCT2r, and EMTh (Fig. 7b). The K_i values for OCT1r [4.2 (3.7–4.7) mM] and EMTh [extrapolated to 13 (11–16) mM] indicate very weak inhibition of these transporters by guanidine. By contrast, the K_i for OCT2r was 0.28 (0.26–0.30) mM, which approximately corresponds to the K_m value. It follows that OCT2r has a clearly higher affinity for guanidine than OCT1r (15 times) or EMTh (46 times). This altered recognition may explain, at least in part, why OCT1r and in particular EMTh do not appreciably transport guanidine.

Uptake of Guanidine Derivatives. The intriguing pattern of guanidine uptake could point to a key difference in the solute recognition sites of OCT2 and EMT. To test this point, radiolabeled solutes with a guanidine moiety, cimetidine and creatinine, were examined for uptake in parallel experiments (Fig. 8, total uptake, top, and uptake relative to MPP^+ uptake, bottom).

Strikingly, with both solutes tested, an identical pattern was recorded, already familiar from guanidine; transport was highest by OCT2r, much lower by OCT1r, and lowest by EMTh. Note however, that uptake by OCT2r of creatinine was, with a factor of about 0.1 relative to MPP^+ uptake, lower by an order of magnitude than uptake of cimetidine. In

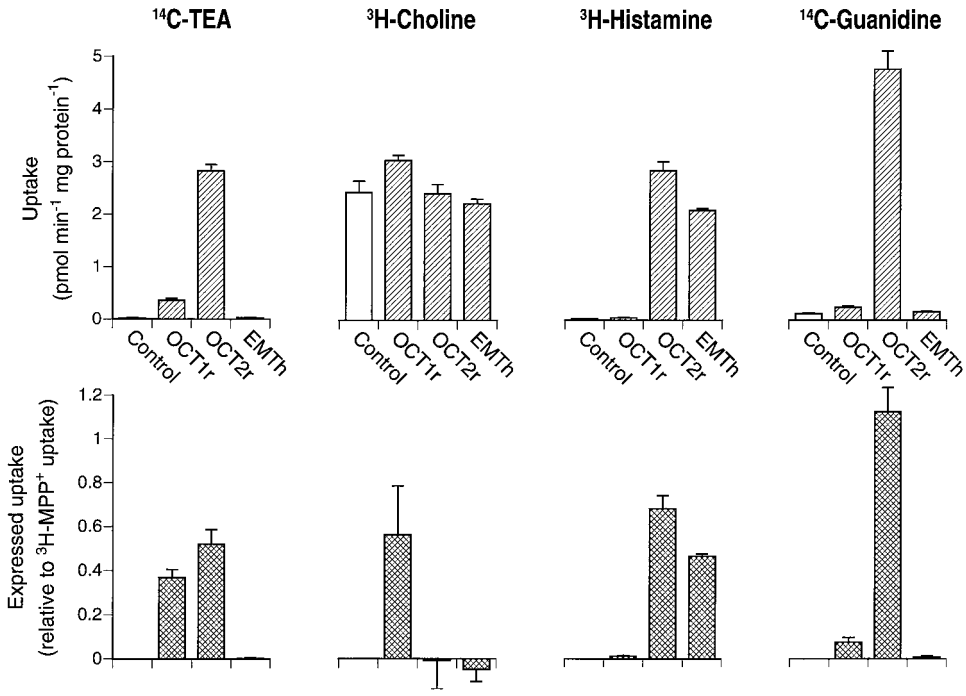

Fig. 4. Comparison of efficiency of transport of TEA, choline, histamine, and guanidine by OCT1r, OCT2r, or EMTh. Control cells had been transfected with vector pcDNA3 alone. Initial rates of transport were determined for the indicated solutes (0.1 μM) and cell lines at 37°C with an uptake period of 1 min. Shown is mean ± S.E.M. (n = 3). Top, original data; bottom, same data after normalization. As an example, total MPP^+ uptake rates from a single experiment were 0.11 ± 0.01 (control), 1.0 ± 0.1 (OCT1r), 5.5 ± 0.6 (OCT2r), and 2.7 ± 0.1 pmol min^{-1} mg $protein^{-1}$ (EMTh).

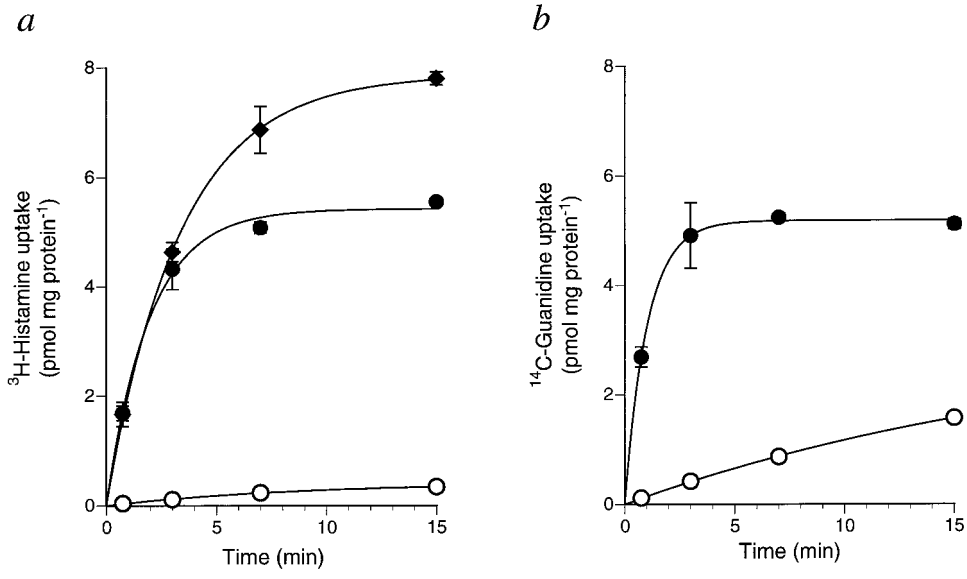

Fig. 5. Time course of uptake of histamine (a) or guanidine (b) into 293 cells expressing OCT2r or EMTh. Cells in dishes expressing OCT2r (a, b; ●) or EMTh (a; ♦) were incubated for the indicated time periods at 37°C with 0.1 μM histamine or guanidine. Control cells (○) had been stably transfected with vector alone. Shown is mean ± S.E.M. (n = 3).

other words, although OCT2r translocates cimetidine very efficiently, it transports creatinine at a much lower rate. In control experiments, uptake of creatine or adenosine by cells expressing OCT1, OCT2, or EMTh was not increased over wild-type cells (data not shown).

Cimetidine uptake mediated by OCT2r was analyzed in more detail. Analysis of the time course of uptake of cimetidine into pcDNA3OCT2r-transfected cells (Fig. 9a) revealed the following rate constants: k_{in} = 26 ± 3 μl min^{-1} mg protein^{-1}, k_{out} = 1.2 ± 0.2 min^{-1}, n = 12 (control cells: k_{in} = 0.86 ± 0.06 μl min^{-1} mg protein^{-1}, k_{out} = 0.48 ± 0.04 min^{-1}, n = 12).

Expressed uptake of cimetidine was saturable (Fig. 9b), with K_m = 21 (18–24) μM and V_{max} = 0.20 ± 0.01 nmol min^{-1} mg protein^{-1}. By contrast, uptake of creatinine by OCT2r did not show saturation up to a concentration of 1 mM (data not shown). This suggests a K_m equal to or higher than 5 mM.

***Trans*-Stimulation Experiments.** The efficiency of guanidine, histamine, and MPP$^+$ to accelerate by counter-transport uptake of MPP$^+$ by OCT2r was examined. Cells expressing OCT2r were preincubated for 20 min in uptake buffer with 1 mM unlabeled solutes. Controls were incubated in uptake buffer alone. After thorough washing, uptake of ^{3}H-labeled MPP$^+$ was measured as usual. Cells preloaded with MPP$^+$ did show a modest, but significant increase in MPP$^+$ uptake relative to control (Fig. 10). On the basis of specific uptake, this *trans*-stimulation amounts to a factor of 1.7. Specific uptake was defined as that fraction of total uptake that was sensitive to 2 μM disprocynium 24 (Russ et al., 1993). By comparison, with a factor of 4.0, guanidine was a better stimulant. With a factor of 3.2, *trans*-stimulation by histamine was intermediate in extent.

Discussion

With the recent cloning of non-neuronal transport proteins for monoamines, it has become possible to determine, by heterologous expression, reliable kinetic parameters for individual transporters. In the present study, stably transfected lines of 293 cells expressing either EMT, OCT2, or OCT1 were examined side by side in uptake experiments with radiolabeled solutes. Two additional members of the amphiphilic solute facilitator family have been claimed to function as organic cation transporters (Tamai et al., 1997; Wu et al., 1998), i.e., OCTN1 and OCTN2. The latter, which

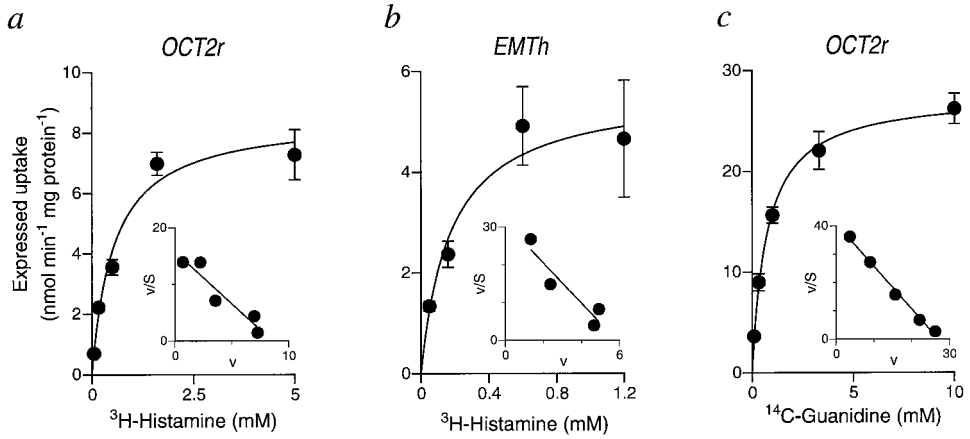

Fig. 6. Saturation of expressed uptake of histamine (a, b) or guanidine (c) into 293 cells expressing OCT2r (a, c) or EMTh (b). An uptake period of 45 s was chosen to approximate initial rates of transport. Shown is mean ± S.E.M. (n = 3). Expressed uptake was calculated by subtraction from total uptake of uptake into cells transfected with vector pcDNA3 alone. This control uptake increased linearly with substrate concentration, slope = 0.233 (a), 0.466 (b), 0.271 (c) μl min^{-1} mg protein^{-1}. Insets: Eadie-Hofstee transformation.

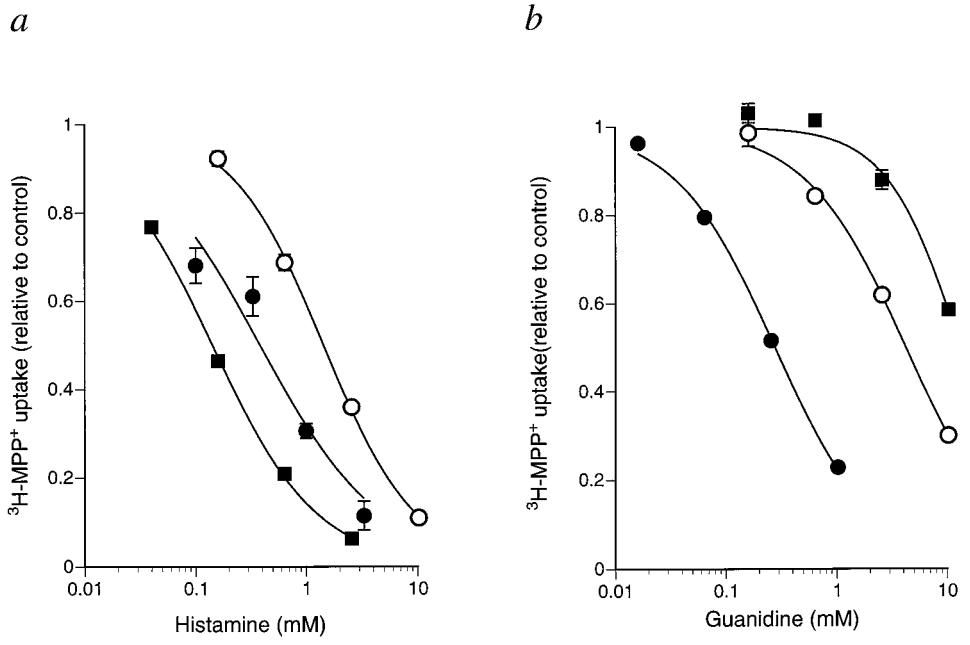

Fig. 7. Inhibition of specific MPP$^+$ uptake into 293 cells expressing OCT1r, OCT2r, or EMTh by histamine (a) or guanidine (b). An uptake period of 1 min was chosen to approximate initial rates of transport. Uptake of MPP$^+$ (0.1 μM) into 293 cells expressing OCT1r (○), OCT2r (●), or EMTh (■) was determined in the presence and absence of histamine (a) or guanidine (b). Shown is mean ± S.E.M. (n = 4) of the uptake in the presence of inhibitor relative to control. Specific uptake was defined as that fraction of total uptake that was sensitive to 2 μM disprocynium24. Hill coefficients were 1.05 ± 0.04, 0.80 ± 0.11, and 0.92 ± 0.02 for inhibition by histamine of OCT1r, OCT2r, and EMTh, respectively, and 0.96 ± 0.05, 0.96 ± 0.03, and 1.32 ± 0.14 for inhibition by guanidine of OCT1r, OCT2r, and EMTh, respectively.

was first published as UST2 (Schömig et al., 1998), has been identified recently as a carnitine transporter (Tamai et al., 1998). Because neither transports MPP^+ or monoamines (data not shown), they were omitted from the present study.

Previous independent work from three laboratories including our own had established, with minor discrepancies, the primary structure of OCT2r (Okuda et al., 1996; Gründemann et al., 1997; Gorboulev et al., 1997). However, in this study, we have presented evidence for the authentic primary structure of OCT2r with a completely different C terminus (Fig. 1). Consequently, all functional assays were performed with authentic OCT2r. To examine whether this substitution of the C terminus affects transport activity, we determined the half-saturating concentration (K_m) for uptake of dopamine (Fig. 3b). The value obtained, 2.3 mM, agrees perfectly with 2.1 mM, the corresponding K_m from a recent report, in which we have shown that (the artificial) OCT2r accepts dopamine as substrate (Gründemann et al., 1998b). Thus, at present there is no evidence that the transport activity of authentic OCT2r is significantly different from the artificial variant.

Efficiency of solute transport is denoted, analogous to catalytic efficiency in the context of enzymology, by k_{cat}/K_m. Based on the familiar Michaelis-Menten equation, this ratio takes into account for a particular transport substrate the affinity (K_m) and turnover number (k_{cat}) of the transporter under study. If in a comparison of various substrates the transporter number (E_{total}) is constant, the transport efficiency may conveniently be expressed as V_{max}/K_m (because $V_{max} = k_{cat} * E_{total}$), or, equivalently, as clearance, which is abstracted as k_{in} from a time course of uptake at a substrate concentration much smaller than K_m. Alternatively, and as practiced in this study, the comparison of transport efficiency may simply be based on initial uptake rates, which are directly proportional to k_{cat}/K_m provided that an identical concentration, much smaller than the respective K_m, is used for every substrate, and E_{total} is constant.

It was the aim of the present study to compare the efficiency of solute transport of three different transporters, each expressed in a separate cell line. In this setting, it becomes necessary to correct the uptake rates for transporter number, which must be expected to vary from one cell line to the other. We performed this normalization by calculating the uptake of a particular substrate relative to MPP^+ uptake (Figs. 4 and 8, bottom), which was measured in paired assays. This approach is based on the observation that OCT1, OCT2, and EMT have virtually identical affinities for MPP^+ (Russ et al., 1992; Gründemann et al., 1994; Martel et al., 1996; this work), and, in the absence of experimental data, on the presumption that turnover numbers are at least similar

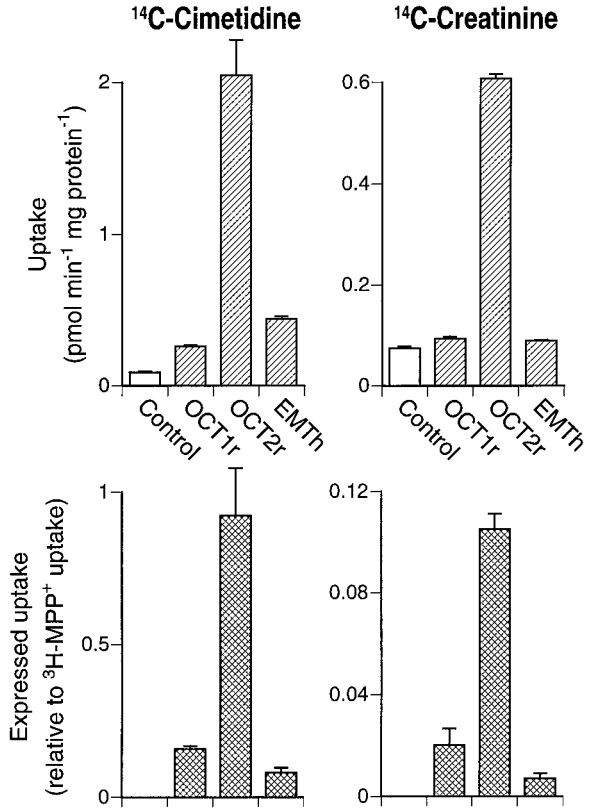

Fig. 8. Comparison of efficiency of transport of cimetidine and creatinine by OCT1r, OCT2r, or EMTh. Shown is mean ± S.E.M. (n = 3). See legend to Fig. 4 for additional information.

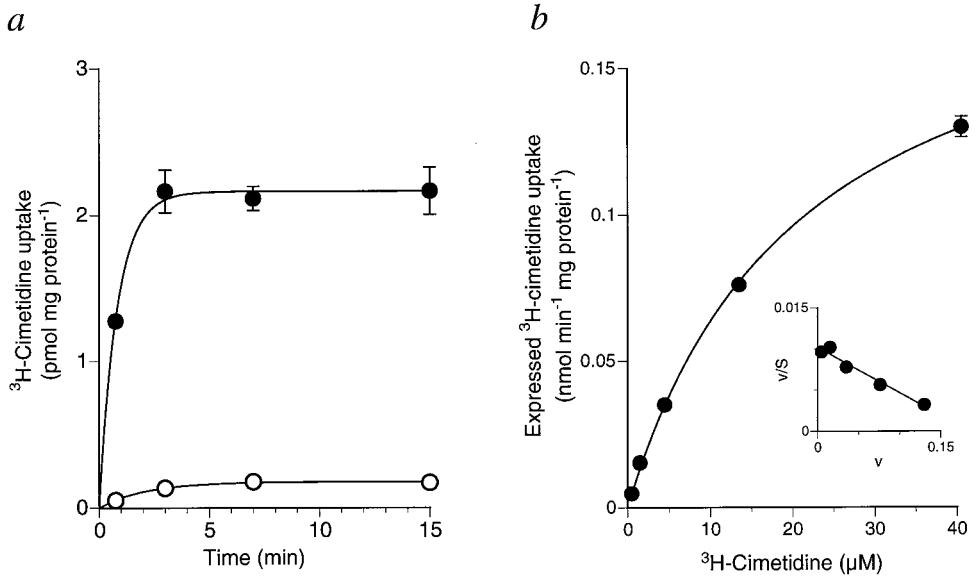

Fig. 9. Time course of uptake of cimetidine (a) and saturation of expressed uptake of cimetidine (b) into 293 cells expressing OCT2r. a, cells in dishes expressing OCT2r (●) were incubated for the indicated time periods at 37°C with 0.1 μM cimetidine. Control cells (○) had been stably transfected with vector alone. Shown is mean ± S.E.M. (n = 3). b, an uptake period of 1 min was chosen to approximate initial rates of transport. Shown is mean ± S.E.M. (n = 3). Expressed uptake was calculated by subtraction from total uptake of uptake into cells transfected with vector pcDNA3 alone. This control uptake increased linearly with substrate concentration, slope = 0.375 μl min^{-1} mg $protein^{-1}$. Inset: Eadie-Hofstee transformation.

because of homologous transporter structures. It then follows from the Michaelis-Menten equation that initial uptake rates are directly proportional to transporter number. The proposed normalization is also useful for comparison of transport efficiencies within a single cell line because it accurately corrects for gradual decline in transporter number over culture time (data not shown).

Our normalized uptake data indicate impressive differences in transport efficiencies between OCT1, OCT2, and EMT (Fig. 4, bottom). With an uptake of about 0.5 relative to MPP^+, TEA was a good substrate for OCT1r and OCT2r. It was not, however, accepted as substrate by EMTh. As expected from a previous report (Schloss et al., 1994), measurement of choline uptake was impaired by a high endogenous uptake activity of the 293 cells. Nevertheless, it is apparent from our data that choline was transported exclusively by OCT1, at a rate similar to TEA. Because of the high endogenous uptake, choline is not a suitable substrate for the characterization of OCT1 function, at least in 293 cells. Histamine was a good substrate, with an uptake of about 0.6 relative to MPP^+, for OCT2 and EMT, but was not transported by OCT1. Finally, guanidine was an excellent substrate for OCT2r, with uptake as high as that of MPP^+. Transport of guanidine by OCT1 was low, and transport by EMTh was negligible. With the guanidine derivatives cimetidine and creatinine, a pattern strikingly similar to guanidine was observed (Fig. 8, bottom); transport was highest by OCT2r, much lower by OCT1r, and lowest by EMTh.

Detailed analysis revealed similar affinities (200–500 μM) of OCT2r and EMTh for histamine (Figs. 6 and 7). This characteristic, and the lack of choline transport confirm the functional similarity of both transporters that was first revealed by remarkably similar inhibition profiles (Schömig et al., 1993). Although the affinity of OCT1r for histamine is only moderately reduced (Fig. 7a), there is virtually no transport of histamine. This indicates that histamine is not compatible with the conformational change of OCT1 that must occur during the transport cycle.

By contrast, guanidine strongly discriminates OCT2 and EMT (Fig. 4). This may be caused, at least partly, by the reduced affinity of EMTh (about 50-fold; Fig. 7b). Guanidine is transported by OCT2r with conspicuously high efficiency, as good as MPP^+. Because the affinity of OCT2r is much smaller for guanidine compared with MPP^+ (about 30-fold), efficient transport of guanidine is due to a high turnover number (k_{cat}), as can be seen from saturation analysis (cf. Figs. 3a and 6c). In other words, OCT2r translocates guanidine much faster across the membrane than other substrates. In accordance with this concept, guanidine clearly was a better driver in the *trans*-stimulation experiment than MPP^+ (Fig. 10).

Our results confirm that EMT is not an organic cation transporter [as the name "OCT3" would suggest (Kekuda et al., 1998)], but rather a transporter for monoamine transmitters (Gründemann et al., 1998a). Of all the solutes tested in the present study, it only transported histamine and cimetidine in significant amounts. It did not, however, accept TEA, choline, guanidine, or creatinine, all of which may be regarded as classical organic cations.

How do the present findings fit with previous studies with membrane vesicles? First, we hypothesize that EMT mediates guanidine/H^+ antiport into vesicles from placenta and intestine. This is based on the demonstration of guanidine transport activity with moderate affinity (K_m around 200 μM) with vesicles from human placenta (Ganapathy et al., 1988) and rabbit intestine (Miyamoto et al., 1988). Because most features were very similar for both mechanisms and inhibition profiles correlate very well, it is conceivable that a single transporter is responsible for both transport activities. Notably, an outwardly directed H^+ gradient produced an "overshoot" in guanidine uptake. In both preparations, there was no uptake of TEA. The renal organic cation/H^+ antiporter transports TEA, whereas the antiporter from placenta does not (Prasad et al., 1992). From the known tissue distributions, the involvement must be excluded of OCT1 and OCT2, but not of EMT.

Second, we hypothesize that OCT2 mediates guanidine/H^+ antiport and organic cation/H^+ antiport into renal brush-border vesicles. This is based on the demonstration of guanidine transport activity with brush-border vesicles from rabbit (Miyamoto et al., 1989) and human kidney (Chun et al., 1997). With rabbit vesicles, the inhibition profile for guanidine uptake correlates almost perfectly with the inhibition profile for TEA uptake (Miyamoto et al., 1989). Additional support for the concept that OCT2 corresponds to the apical organic cation/H^+ antiporter comes from a number of studies on renal cimetidine transport. The K_m of OCT2r for cimetidine, 21 μM (Fig. 9b), agrees with previous work with isolated rat proximal tubular cells (K_m = 7 μM; Boom and Russel, 1993), with rabbit renal brush-border vesicles (K_m = 5 μM; Gisclon et al., 1987), and with LLC-PK_1 cells (K_m = 32 μM; Bendayan and Silverman, 1994). Cimetidine/H^+ antiport was demonstrated with rabbit brush-border membrane

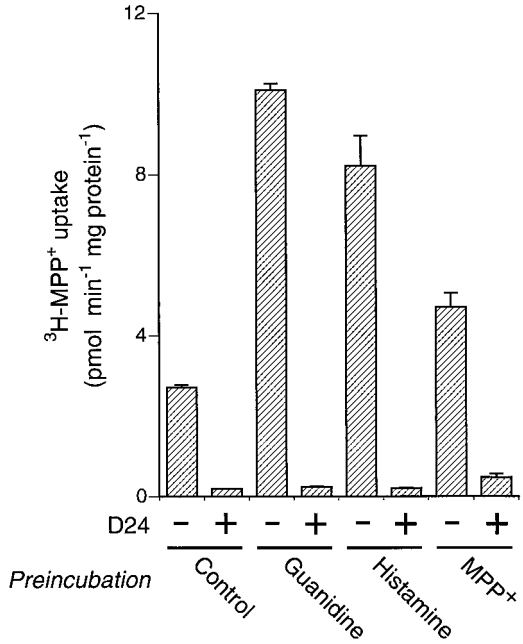

Fig. 10. *Trans*-stimulation of MPP^+ uptake into 293 cells expressing OCT2r. Shown is mean ± S.E.M. (n = 3) of total uptake. Cells grown in dishes were preloaded by preincubation for 20 min in buffer (control), supplemented, as indicated, with 1 mM unlabeled substrate. After washing four times with 3 ml ice-cold buffer to remove substrate from extracellular space, uptake of MPP^+ (0.1 μM, 45 s) was measured. Parallel assays marked D24 + were performed with 2 μM disprocynium 24 both in preincubation and uptake buffers to inhibit OCT2r and to estimate specific uptake.

vesicles (Gisclon et al., 1987). With the same model, cimetidine/H^+ antiport was inhibited with low potency by creatinine (Gisclon and Giacomini, 1988). With isolated perfused rabbit tubules that were comprised of S_2 and S_3 segments, secretion of cimetidine from bath to lumen was inhibited by creatinine (McKinney et al., 1981). This also conforms to a previous study in which we have shown that OCT2r is expressed exclusively in S_3 segments (Gründemann et al., 1998b). In a clearance study with human subjects, secretion of creatinine was decreased by cimetidine (Burgess et al., 1982). With rabbit brush-border vesicles, TEA inhibited cimetidine/H^+ antiport, and cimetidine inhibited TEA/H^+ antiport (Takano et al., 1985). Together, these observations suggest that TEA, cimetidine, and creatinine share a common carrier, and that cimetidine is recognized with much higher affinity than creatinine. This perfectly fits our data and suggests that OCT2 may be involved in the clearance of creatinine.

In vesicle studies, *trans*-stimulation by a H^+ gradient constitutes the hallmark of the apical organic cation transporter, which, according to our hypothesis, corresponds to OCT2. However, in intact cells, OCT2 and EMT are pH- and potential-dependent, but a proton gradient does not *trans*-stimulate transport (Schömig et al., 1992; Gründemann et al., 1997; Kekuda et al., 1998; data not shown). To resolve this contradiction, we propose that proton antiport in vesicle studies is due to the use of unphysiological buffers. Support comes from the observation that inorganic cations markedly reduce the overshoot phenomenon (Miyamoto et al., 1989). EMT, the close functional relative of OCT2, might show the same peculiarity, because inorganic cations such as K^+ and Na^+ clearly inhibit guanidine uptake with vesicles from placenta and intestine (Ganapathy et al., 1988; Miyamoto et al., 1988). Finally, the use of buffers largely devoid of inorganic cations may also explain why there was, by contrast to our data, guanidine transport at all with vesicles that presumably contain EMT.

It is presently unclear which transporter is responsible for uptake of guanidine into cultured HeLa cells (Nair, 1987; 1988) and JAR cells (Zevin et al., 1997).

By analogy to selective receptor agonists, selective transport substrates significantly extend pharmacological profiles based on inhibitors. We have identified substrates that allow functional discrimination of non-neuronal monoamine transporters OCT1, OCT2, and EMT. Although these transporters show some overlap in substrate specificity, and many of the large hydrophobic inhibitors such as quinine are effective to similar extents, clear-cut differences were observed for efficiency of transport of cimetidine, creatinine, guanidine, histamine, choline, and TEA. These substrates reveal key differences in solute recognition and turnover and thus challenge the concept of "polyspecific" organic cation transporters.

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