Guanidine Transport in a Human Choriocarcinoma Cell Line (JAR)

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Purpose. Many endogenous substances and xenobiotics are organic cations. Transplacental transport of organic cations is an important determinant of the delivery of these compounds to the fetus. The aim of this study was to determine the mechanisms of organic cation transport using the human choriocarcinoma cell line (JAR) as a model system with [^{14}C]guanidine as a ligand.

Methods. Uptake studies of [^{14}C]guanidine were carried out in JAR cell monolayers on day 2 after plating.

Results. [^{14}C]guanidine uptake was temperature dependent, saturable (K_m = 167 μM) and inhibited by many organic cations including amiloride, cimetidine, quinine, quinidine and nicotine. [^{14}C]guanidine uptake exhibited a counterflux phenomenon indicative of a carrier-mediated process. The uptake of [^{14}C]guanidine was sodium and pH-independent and could be driven by an inside-negative membrane potential difference.

Conclusions. This is the first demonstration of an electrogenic guanidine transporter in a human cell culture model. This transporter may play a role in the transplacental transport of many clinically used drugs and xenobiotics.

KEY WORDS: organic cation transport; placenta; guanidine; JAR cells.

INTRODUCTION

Many clinically used drugs, including a number of antihypertensive agents, antiarrhythmic agents, antihistamines and antidepressants are organic cations. These compounds may be used or indicated for use during pregnancy. Transplacental flux of organic cations is a major determinant of fetal exposure to these compounds and may involve specific transporters located in the placental plasma membranes.

An organic cation-proton antiporter for guanidine and 5-(N-methyl-N-isobutyl)amiloride (MIBA) was demonstrated in human placental microvillous membrane vesicles (1,2). This transporter is distinct from the well-described renal organic cation-proton antiporter in terms of its substrate selectivity. Notably, no tetraethylammonium (TEA) transport could be demonstrated in the microvillous membrane vesicles. Guanidine-selective organic cation transporters which either exclude or have low affinity for TEA have also been described in renal and intestinal brush border membrane vesicles from rabbit (3,4). To date, a cell culture model for guanidine selective organic cation transporters has not been described.

The focus of this study was to develop a cell culture model for guanidine selective organic cation transport. Cultured cells are advantageous for studies of regulation and electrophysiology of transport processes. We chose the human choriocarcinoma cell line (JAR) as a model system for these studies. JAR cells can be maintained in a continuous culture and form monolayers (5,6). Several transporters, including transporters for thyroid hormone, serotonin, taurine and glycine, have been identified and characterized in JAR cells (7–11). Because several of these transporters are present in human placenta (12,13), JAR may be a suitable model for studying biologically relevant placental transport processes.

MATERIALS AND METHODS

Cell Culture

JAR cells were obtained from the Cell Culture Facility at the University of California, San Francisco and maintained in culture at 37°C in a humidified 5% CO_2, 95% air atmosphere. The growth medium was RPMI-1640, supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% amphotericin B. All studies were performed in cells between passages 725 and 745. For transport studies in monolayers, the cells were subcultured in 12-well plates following trypsinization with 0.05% trypsin containing 0.02% EDTA. The seeding density was 0.5 * 10^6 cells/well (1.3 * 10^5 cells/cm^2). The medium was changed after 24 hours and the monolayers were used for experiments on day 2 after plating.

Uptake Measurements

To study the uptake of [^{14}C]guanidine in the JAR cell monolayers, each monolayer was rinsed three times with a buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM Hepes/TRIS (pH 7.4). We compared the uptake of guanidine using either 25 mM Hepes/TRIS (pH 7.4) or 25 mM Hepes/NaOH (pH 7.4), and found no effect of TRIS on the uptake (96.5 ± 18.2 pmol/mg protein/3 min vs 92.2 ± 3.7 pmol/mg protein/3 min). To initiate uptake, 0.5 ml of the buffer containing 20 μM of [^{14}C]guanidine (uptake medium) was added to each well and the wells were incubated at room temperature for the given time (three minutes for most experiments). The amount of tracer added to each well was 0.54 μCi. The uptake was stopped by aspirating the uptake medium and washing the wells three times with ice-cold buffer. The cells were solubilized in 1 ml of 0.5% Triton X-100, and an aliquot of the solubilized cells was transferred to scintillation vials to determine radioactivity. Inhibition studies were carried out by adding various concentrations of the unlabeled compounds to the uptake medium. For counterflux studies, JAR cells were preincubated for 30 minutes with or without unlabeled guanidine (1 mM), and the uptake of [^{14}C]guanidine was determined at 3 min. For kinetic studies, the uptake of [^{14}C]guanidine was determined at 3 min in uptake medium containing various concentrations of guanidine (range 10 μM to 1 mM).

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Protein Assay

The protein concentration in cell monolayers was measured by the method of Bradford, using the Bio-Rad reagent (14). Bovine serum albumin was used as a standard.

Studies with Ionophores

Following preincubation for 20 min with either ouabain (1 μM or 1 mM) or monensin (10 mg/l), [^{14}C]guanidine uptake was determined at 3 min. Control cells were incubated in buffer. For studies of membrane potential, valinomycin (1 μM) dissolved in ethanol was added to an uptake medium containing either 4.5 mM KCl (i.e. in the presence of an outwardly—directed K^+ gradient) or 145 mM KCl (i.e., voltage clamped). The same amount of ethanol was added to control cells. Following preincubation with 2,4-dinitrophenol (DNP) (250 μM) for 30 minutes, the uptake of [^{14}C]guanidine at 3 min was determined in uptake medium containing DNP (250 μM).

Effect of pH on [^{14}C]Guanidine Uptake

For pH studies, the uptake of [^{14}C]guanidine was determined at 3 min in buffer at pH 5, 7.4 or 8. To evaluate the effect of an acidified intracellular pH, we preincubated the cells with NH_4Cl (20 mM) for 20 min, then determined [^{14}C]guanidine uptake at 3 min.

Thin Layer Chromatography

Thin layer chromatography (TLC) of [^{14}C]guanidine associated with the cells at 3 min was carried out using methanol, chloroform and ammonium hydroxide (2:1:0.2) as a mobile phase on pre-coated cellulose plastic sheets.

Data Analysis

In general, each data point was determined at least in triplicate for each experiment. All the experiments, except the experiment evaluating the effect of days in culture on guanidine uptake, were repeated at least once on a different day using a different cell passage. Inhibitable guanidine uptake was calculated by subtracting the uptake in the presence of unlabeled guanidine (1 mM) from the total [^{14}C]guanidine uptake in the absence of unlabeled guanidine. The data are presented as mean ± standard deviation (S.D). Statistical significance was determined by the unpaired Student's *t* test. Results were considered to be statistically different at a probability of less than 0.05 ($p < 0.05$).

Materials

[^{14}C]Guanidine (specific activity 56 mCi/mmol) was purchased from Moravek. The following chemicals were purchased from Sigma: guanidine, amiloride, cimetidine, clonidine, quinine, quinidine, valinomycin, ouabain, monensin, choline, NMG, TEA, nicotine and procainamide. The protein assay dye reagent was from Bio-Rad. TLC plates were from EM reagents. Falcon culture plates were used for most experiments. Cell culture supplies were purchased from the Cell Culture Facility at UCSF.

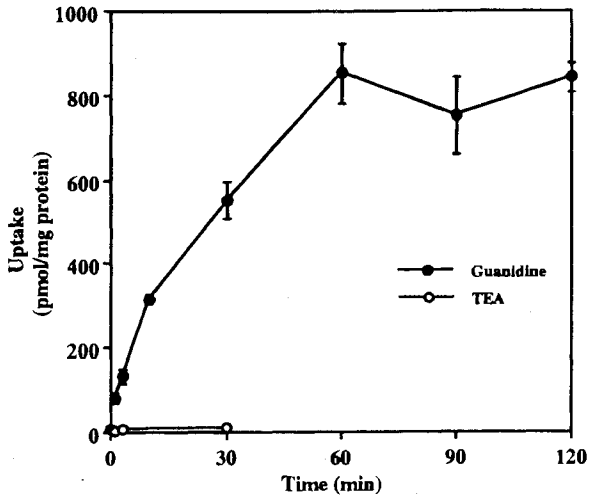

Fig. 1. Time course of guanidine and TEA uptake in JAR cells. The uptake of [^{14}C]guanidine (20 μM) and of [^{14}C]TEA was measured at room temperature (24°C). Data represent the mean ± SEM of determinations in 6–11 wells in 3 different cultures.

RESULTS

[^{14}C]Guanidine accumulated with time in the JAR cell monolayers, reaching equilibrium at approximately 60 min (Fig. 1). In contrast, the uptake of [^{14}C]TEA was negligible (Fig. 1) and not inhibited by 5 mM unlabeled TEA. [^{14}C]guanidine uptake was markedly inhibited by unlabeled guanidine and was also temperature dependent (Fig. 2). Uptake in the presence of 1 mM and 5 mM unlabeled guanidine was similar (17.1 ± 3.1 and 17 ± 6.5 pmol/mg protein/3 min, respectively); therefore, a concentration of 1 mM was used for inhibition of specific uptake in subsequent experiments. The total and the inhibitable uptake of guanidine was highest on the second day after plating

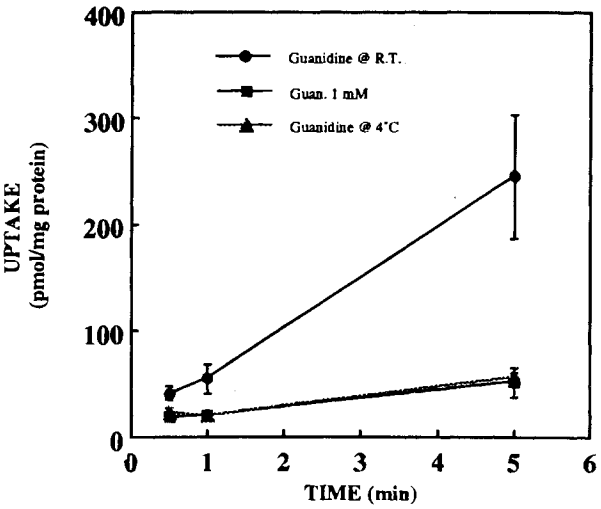

Fig. 2. The effect of temperature on guanidine uptake in JAR cells. Uptake of [^{14}C]guanidine was measured at room temperature (24°C) with (squares) and without (closed circles) unlabeled guanidine (1 mM) and at 4°C (triangles). Data represent mean ± S.D. of determinations in 7 wells in 2 different cultures. At all the time points the uptake at 4°C was significantly different from the control at 24°C ($p < 0.05$).

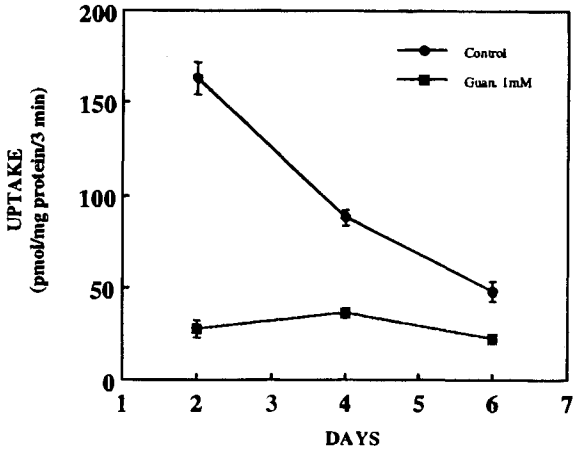

Fig. 3. Effect of days in culture on guanidine uptake and inhibition in JAR cells. The 3 minute uptake of [^{14}C]guanidine was measured in the presence (squares) and absence (closed circles) of 1 mM unlabeled guanidine. Data represent mean ± S.D. of determinations in 4 wells.

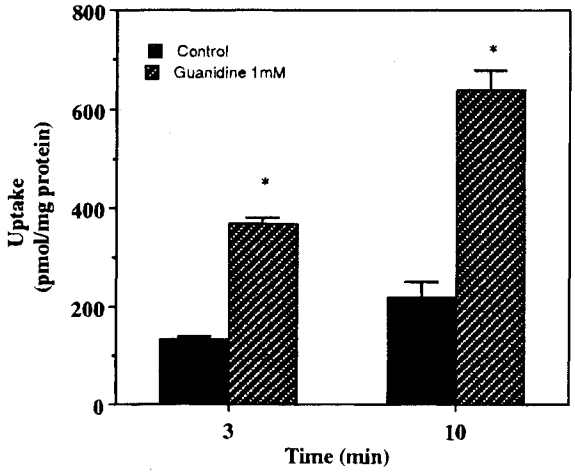

Fig. 5. Trans-stimulation of [^{14}C]guanidine uptake by unlabeled guanidine. Uptake of [^{14}C]guanidine was measured at 3 minutes in control cells or in cells which were preincubated with 1 mM unlabeled guanidine for 30 minutes. Data represent mean ± S.D. of determinations in 7 wells in 2 different cultures . The asterisk indicates significant difference from the control ($p < 0.05$).

and decreased thereafter (Fig. 3). Therefore, the uptake studies were conducted on day 2 after plating.

The saturability of guanidine uptake was determined by measuring the rate of [^{14}C]guanidine uptake at three minutes, a time at which uptake is linear (Fig. 1), versus guanidine concentration. The following equation was used to fit the kinetic data:

$$\text{rate of uptake} = (V_{max} * C)/(K_m + C) + C * K_{ns},$$

where V_{max} and K_m are Michaelis-Menten constants, C is the guanidine concentration in the medium and K_{ns} is a first-order rate constant describing the non-saturable process. The mean data from four separate wells were fit by computer. A K_m of 167 ± 79 μM (mean ± S.E.), V_{max} of 499 ± 145 pmol/mg protein/3 min. and K_{ns} of 0.5 ± 0.1 were estimated (Fig. 4). Saturable uptake accounts for about 87% of total guanidine uptake at a concentration of 20 μM. Thin layer chromatography (TLC) analysis demonstrated that at 3 minutes (and at 1 hr) guanidine was not significantly metabolized (<10%, data not shown).

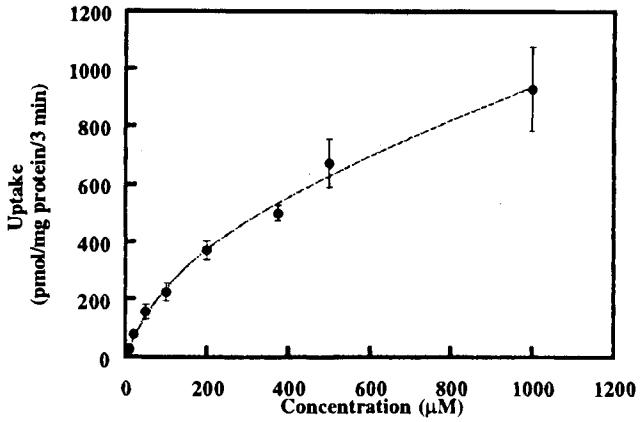

Fig. 4. Effect of concentration on the rate of guanidine uptake in JAR cells. Uptake of [^{14}C]guanidine was measured at each concentration at room temperature (24°C). Data were fit to an equation: rate of uptake = $(V_{max} * C)/(K_m + C) + C * K_{ns}$. K_m of 167 μM and V_{max} of 499 pmol/mg protein/3 min were estimated. Data represent mean ± S.D. of determinations in 7 wells in 2 different cultures.

The uptake of [^{14}C]guanidine was stimulated significantly ($p < 0.05$) in cells that were pre-loaded with unlabeled guanidine (1 mM) indicating a counterflux phenomenon (Fig. 5). As shown in Table I, various organic cations including amiloride, choline, clonidine, procainamide, quinine, quinidine, verapamil, TEA and nicotine significantly inhibited [^{14}C]guanidine uptake in JAR monolayers. The inorganic cations K^+ and Li^+ at concentrations of 10 and 1 mM respectively, did not inhibit the uptake. Para-aminohippuric acid (PAH), an organic anion, as well as lysine, a basic amino acid and taurine, a neutral amino acid, also did not affect [^{14}C]guanidine uptake indicating that the process is selective for organic cations.

[^{14}C]Guanidine Transport—Driving Force

2,4-Dinitrophenol (DNP), a metabolic inhibitor, did not affect [^{14}C]guanidine uptake (Table 2). Furthermore, there was no difference in the uptake at different pH's (the uptake values were 162 ± 19.8, 149 ± 11.9 and 143 ± 9.32 pmol/mg protein at pH 5, 7.4 and 8, respectively). In addition, no significant difference in [^{14}C]guanidine uptake was found after acid-loading the cells (121 ± 10.6 pmol/mg protein for control and 112 ± 6.17 pmol/mg protein for NH_4Cl loading). However, because we did not measure the intracellular pH, we are unsure as to whether the NH_4Cl produced significant intracellular acidification.

To determine the effect of Na^+ on guanidine transport monensin and ouabain were used (Table 2). Monensin, a sodium ionophore, had no effect on [^{14}C]guanidine uptake suggesting that the process is Na^+-independent. Consistent with a Na^+-independent process, ouabain (1 μM) also had no effect on the inhibitable guanidine uptake (Table 2). However, at a concentration of 1 mM ouabain reduced [^{14}C]guanidine uptake to 63% of control.

Valinomycin (1 μM) with different K^+ concentrations was used to either abolish the membrane potential or to increase the

Table 1. Effect of Inhibitors on [^{14}C]Guanidine Uptake in JAR

Inhibitor	[^{14}C]Guanidine uptake	
	pmol/mg protein/3 min	% control
Control†	106 ± 35.5	100
Guanidine†	33.6 ± 7.8*	32
Amiloride	19.8 ± 7.9*	18
Choline	12.8 ± 2.1*	12
Clonidine	29.5 ± 3.7*	28
Mecamylamine	89.2 ± 10.5	84
Procainamide	26 ± 4.8*	24
Quinidine	17.6 ± 3.7*	16
Quinine	16.6 ± 4.9*	16
Verapamil	27.4 ± 6.9*	26
TEA 5mM	42.5 ± 2.5*	40
NMG	66.7 ± 3*	63
Lithium	83.7 ± 3.9	79
Lysine	90.9 ± 2.5	86
KCl 10 mM	127 ± 7.4	120
Taurine	104 ± 8.6	98
PAH	96.5 ± 10.4	91
Control¶	141 ± 1.97	100
Cimetidine	83 ± 9.3*	59
(−)Nicotine	64.5 ± 5.6*	46
(+)Nicotine	83.9 ± 7*	59

Note: Data are mean ± SD of determinations in 3–6 wells. †Control and Guanidine values represent mean ± S.D. of data obtained from several experiments in total of 17 wells. ¶Control value for the inhibition experiment with nicotine and cimetidine. The asterisk indicates significant difference from the control ($p < 0.05$); statistical significance was determined by comparing each inhibitor to its control obtained in the same experiment. Uptake of [^{14}C]guanidine was measured at 3 minutes in the presence of various inhibitors. The concentration of each inhibitor except KCl and TEA was 1 mM. TEA = tetraethylammonium; NMG = N-methylglucamine; PAH = para-aminohippurate.

Table 2. Effect of Ionophores on [^{14}C]Guanidine Uptake in JAR

Ionophore	[^{14}C]Guanidine uptake		
	Total (pmol/mg protein/3 min)	Total (% control)	Specific (% control)
Control†	131 ± 10	100	100
Ouabain 1 μM	115 ± 15	87.9	90 ± 9
Control‡	86.2 ± 8.4	100	100
Ouabain 1 mM	56.1 ± 4.4*	65	63 ± 9*
Control¶	159 ± 15.9	100	100
Valinomycin 1 μM	203 ± 13.6*	127	125 ± 11*
Valinomycin/KCl	122 ± 9.2*	75	56 ± 11*
Monensin 10 mg/l	173 ± 20	109	113 ± 11
DNP 250 μM	152 ± 20	95	98 ± 10

Note: †Control value for the experiments with ouabain 1 μM; ‡Control value for the experiments with ouabain 1 mM; ¶Control value for the experiments with valinomycin, monensin and DNP. Data are means ± SD of determinations made in 6–9 wells in 2–3 different cultures. Specific uptake represents total uptake minus the uptake in the presence of unlabeled 1 mM guanidine. The asterisk indicates significant difference from the control ($p < 0.05$). Uptake of [^{14}C]guanidine at 3 minutes was measured after the cells were preincubated for 20 minutes with ouabain at two concentrations (1 μM and 1 mM), monensin (10 mg/l) or DNP (250 μM). No preincubation was carried out for valinomycin. All the studies except for valinomycin/KCl were carried out in an uptake medium containing 4.5 mM KCl and 140 mM NaCl; the valinomycin/KCl uptake was in an uptake medium containing 145 mM KCl. DNP is 2,4-dinitrophenol.

potential difference. In the presence of an outwardly-directed K^+ gradient and valinomycin (inside negative membrane potential) a significant increase in the inhibitable guanidine uptake was observed (Table 2). In the absence of a K^+ gradient (no membrane potential), [^{14}C]guanidine uptake was significantly decreased (Table 2). The uptake was also significantly inhibited by clamping membrane potential with extracellular K^+ (145 mM) even in the absence of valinomycin (53 ± 12% of control).

DISCUSSION

This is the first demonstration of a broadly—selective organic cation transporter in a human placental cell line. Using guanidine, an endogenous organic cation, as a ligand, we identified an organic cation transporter in the JAR cell line which is sensitive to temperature, saturable and can be inhibited by various organic cations, including choline, NMG, quinine, quinidine, amiloride, cimetidine, nicotine and TEA. The organic anion, PAH, inorganic cations K^+ and Li^+ and a basic amino acid lysine, did not inhibit guanidine uptake, suggesting that this transporter is selective for organic cations. This inhibition pattern differs from that found previously by Ganapathy *et al.* (1) in placental microvillous membrane vesicles. For example, Ganapathy *et al.* observed no inhibition of guanidine uptake by TEA (10 mM), cimetidine (5 mM), choline (10 mM) and NMG (10 mM). These data suggest that the transporter in JAR for organic cations differs from the organic cation transporter characterized by Ganapathy *et al.* (1).

The transport of guanidine across JAR cell monolayers was saturable, with a K_m of 167 μM. Trans-stimulation of guanidine transport (counterflux) was observed in cells preloaded with unlabeled guanidine. The presence of a counterflux phenomenon suggests that guanidine uptake in JAR cell monolayers is a carrier-mediated process.

To examine the role of membrane potential difference in driving the uptake of guanidine, we conducted experiments with valinomycin. Valinomycin, a potassium ionophore, in the low μM concentration range in the presence of an outwardly-directed K^+ gradient hyperpolarizes the cells (15, 16); when there is no K^+ gradient, the cell is depolarized (17). When the cells were hyperpolarized, specific guanidine uptake was significantly increased (25%) whereas in depolarized cells the uptake was significantly reduced (44%) (Table 2). These data suggest that guanidine transport is dependent, at least in part, upon membrane potential.

The observation that the metabolic inhibitor, 2,4-dinitrophenol (DNP), did not inhibit guanidine uptake, is consistent with a membrane potential-dependent process. It was found that in trophoblast cells the addition of metabolic inhibitors for 15–60 minutes only caused a change of approximately 15% in the membrane potential (17). In addition, we were unable to drive guanidine transport by imposing either a pH gradient or an inwardly-directed Na^+ gradient. Furthermore, addition of ouabain (1 μM), which abolishes the Na^+ gradient, and monensin, a sodium ionophore, did not significantly affect guanidine

uptake. The observation that a pH gradient did not drive guanidine transport in JAR provides additional evidence that the guanidine transporter in JAR differs from the previously described guanidine-proton antiporter in human placental membrane vesicles (1).

Our findings with ouabain (Table 2) are also consistent with a potential driven process. Although Na^+/K^+ ATPase plays a role in maintaining membrane potential, low concentrations of ouabain (1 μM), which have been shown to inhibit Na^+/K^+ ATPase in human cell lines (18–20) cause only a small reduction in membrane potential in some cells, including cytotrophoblast (17) and many malignant cells (20), whereas higher concentrations may have non-specific cytotoxic effects (11). Interestingly, TEA inhibited guanidine uptake, while no uptake of [^{14}C] TEA was found (Fig. 1). This may be due to the fact that TEA is an inhibitor of K^+ channels (21) and thus can cause dissipation of the inside-negative membrane potential. Alternatively, TEA may be an inhibitor, but not a substrate of the transporter.

In conclusion, our data provide the first evidence for a broadly selective, electrogenic guanidine transporter in a cell culture model. The transporter differs from the guanidine transporters described in microvillous membrane vesicles in placenta, intestine and kidney (1, 3, 4) since it does not appear to be an organic cation-proton antiporter and has a different substrate selectivity. It also appears to be different from the choline transporter in microvillous placental membrane vesicles characterized by Grassl (22) based on substrate selectivity. Namely, procainamide did not inhibit choline uptake in the vesicles, whereas procainamide inhibited guanidine uptake in JAR. JAR cells do not exhibit the polarization of differentiated epithelial cells which precludes assignment of the transporter to the brush-border or basolateral membrane domain (23). In renal proximal tubules an organic cation-proton antiporter is present on the brush border membrane and a potential-sensitive organic cation transporter is present on the basolateral surface (24–30). Although speculative, it is possible that the transporter in JAR cells may correspond to a basolateral placental transporter, consistent with the transepithelial flux model of organic cation secretion in the proximal tubule. Thus, the transporter we characterized may be involved in the excretion of organic cations from the fetus. This transporter may play a role in the transplacental disposition of clinically used cationic drugs.

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