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Cellular transport processes of aminoguanidine, a nitric oxide synthase inhibitor, in the opossum kidney cell culture line

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Abstract

Aminoguanidine has potential pharmacologic utility for diabetes and nitric oxide — mediated inflammation. Because aminoguanidine is positively charged at physiologic pH ($pK_a \sim 10$), it is unlikely that simple diffusion is a predominant mechanism for cellular penetration. This study sought to determine the transport processes by which aminoguanidine, a cationic compound, traverses across cellular membranes. In cultured opossum kidney (OK) cell monolayers, aminoguanidine transport involved both saturable and non-saturable diffusion processes. At passage numbers below 67, the observed V_{max} and K_m for saturable influx were significantly lower than that observed at passages greater than 79 (V_{max}: low passage, 21.2 ± 7.8 pmol/(min∗mg protein), $n = 3$; versus high passage, 129.7 ± 24.3 pmol/(min∗mg protein), $n = 3$, $P < 0.05$; K_m: low passage, 23.7 ± 10.8 μM, $n = 3$; versus high passage, 101.7 ± 5.6 μM, $n = 3$, $P < 0.05$; mean ± S.E.M.). Nonsaturable processes were not statistically different (k_{ns}: low passage, 1.6 ± 0.1 pmol/(min∗mg protein∗μM), $n = 3$; high passage, 1.1 ± 0.2 pmol/(min∗mg protein∗μM) $n = 3$). Saturable influx was temperature dependent, and independent of ATP energy, sodium gradients or changes in membrane potential. Other organic cations competitively inhibited and *trans*-stimulated saturable influx. Aminoguanidine influx was increased in the presence of an outwardly-directed proton gradient and was inhibited in the presence of an inwardly-directed proton gradient. Correspondingly, aminoguanidine efflux was *trans*-stimulated by aminoguanidine and guanidine. In summary, OK cell cultures at high passage numbers (> 79) express a saturable, bi-directional carrier-mediated process to transport aminoguanidine across cellular membranes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Aminoguanidine; Cell culture; Guanidine; Organic cation; Transport; OK cell culture

1. Introduction

Transport proteins evolved out of the necessity to shuttle critical, hydrophilic nutrients or xenobi-

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otics (such as sugars, amino acids, nucleosides, organic acids and organic bases) across cellular membranes throughout the body. Such transport proteins are primarily localized to organs and tissues involved in xenobiotic absorption, distribution, metabolism or elimination, e.g. renal tubules, liver canilicular membranes, intestinal bush border membranes, cerebrovascular endothelial cells of the blood-brain barrier and erythrocyte cellular membranes. Saturation of a transport protein, either by high drug concentrations or drug competition with endogenous compounds, may be a source of nonlinear drug pharmacokinetics. Depending on the nature of the drug transporter and the nonlinear pharmacokinetic process, pharmacodynamically relevant drug concentrations may be super or sub-therapeutic, potentially leading to a therapeutic failure.

Many drugs and endogenous compounds are organic cations at physiological pH. Recent advances in transport research have identified several important organic cationic transport proteins: OCT-1, -2 and a guanidine transporter. The rat OCT1 (rOCT1) transporter has a broad substrate specificity and utilizes electrochemical gradients as a driving force for transport. rOCT1 is expressed in rat liver, small intestines, colon and kidney, with a predominant basolateral localization in the proximal tubule (Grunderman et al., 1994). The rat OCT2 (rOCT2) transporter is 67% identical to rOCT1, has a broad substrate specificity and is expressed primarily in the kidney (Okuda et al., 1996). To date, there are two cloned human organic cationic transporters, hOCT1 and hOCT2 (Gorboulev et al., 1997; Zhang et al., 1997b). Although hOCT1 shares $\sim 78\%$ sequence similarity to rOCT1, there are significant differences in tissue distribution and functionality among the different isoforms. rOCT1 and hOCT2 are localized in the kidney, whereas hOCT1 is primarily localized in the liver. Moreover, large, bulky organic cations are less potent inhibitors of the hOCT1 transporter.

In contrast to the OCT transport proteins, the recently identified guanidine transporter relies on a proton exchange mechanism that is independent of membrane potential. The guanidine transporter is localized to renal (Miyamoto et al., 1989; Chun et al., 1997), intestinal (Miyamoto et al., 1988) and human placental (Ganapathy et al., 1988; Prasad et al., 1992) membranes. The well known OCT substrates, tetraethylammonium (TEA) and *N*-methylnicotinamide are not typical substrates for the guanidine transporter, although there is some overlap in substrate specificity, because other organic cations inhibit both the OCT and guanidine transporters (Miyamoto et al., 1989; Chun et al., 1997).

Renal epithelial cells express many transport systems for organic anions, cations, and endogenous compounds, such as amino acids, glucose and nucleosides (Giacomini et al., 1988; Murer and Biber, 1993; Bendayan, 1996). The proximal renal tubule expresses several distinct transport proteins which may have affinity for guanindino compounds, e.g. a general organic cation/proton antiporter (characterized by the substrates TEA and *N*-methylnicotinamide), a y^+ transporter for the cationic amino acids arginine and lysine, and a guanidine/proton antiporter (Holohan and Ross, 1980, 1981; Hammerman, 1982; Inui et al., 1985; Miyamoto et al., 1989; Yuan et al., 1991). The opossum kidney (OK) cell culture line is a representative model of renal proximal tubule transport processes (Malmström et al., 1987; Schwegler et al., 1989; Yuan et al., 1991). This cell line was used extensively to characterize organic cation transport typified by the model substrate, TEA (Yuan et al., 1991). It is presently unknown whether the OK cell line expresses a unique guanidine transporter.

Aminoguanidine (AG) has potential pharmacologic utility as an inhibitor of (a) advanced glycosylation processes in diabetes (Brownlee, 1989; Edelstein and Brownlee, 1992; Makita et al., 1992); and (b) pathological nitric oxide (NO) production by inducible nitric oxide synthase during neuroinflammation (Boje, 1995, 1996). Because AG is positively charged at physiologic pH ($pK_a \sim 10$), it is unlikely that simple diffusion is a predominant mechanism for cellular penetration. Pharmacokinetic literature data suggest that AG undergoes active renal secretion in man, because AG renal clearance was ~ 2.2 times greater than the glomerular filtration rate (Foote et al., 1995). Since AG is an organic cation and a prototype guanidinium compound, secretion is likely to oc-

cur through the organic cation-proton and/or guanidine-proton exchange systems. Utilization of transport processes at the renal tubules may have an impact on the pharmacokinetics or pharmacodynamics of AG, depending on the desired serum concentration for therapeutic activity. The overall goal of the present investigation was to mechanistically test the hypothesis that the organic cation, AG, utilizes transport processes distinct from diffusion in the OK renal cell line.

2. Materials and methods

2.1. Cell culture

OK cells were obtained on the 36th passage from ATCC and were studied from passage numbers 44–108. The cell line was grown in Eagle's MEM (Earle's salts) supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cultures were grown and maintained using standard protocols. Approximately 1 week prior to study, confluent cultures were subcultured on 6-well plates at a seeding density of 4×10^4 cells/cm^2. Cultures were fed fresh media every 3rd day and on the day preceding transport studies.

2.2. General protocol for transport studies

Cell culture transport experiments were performed as described by Yuan et al. (1991) with minor modifications. On the day of the study, the media was aspirated and cultures were rinsed three times with Dulbecco's phosphate buffered saline (PBS), (components in millimolar concentrations: KCl, 2.7; KH_2PO_4, 1.5; NaCl, 138; Na_2HPO_4, 8; pH 7.4) to remove residual media. Cellular uptake of the probe of interest was initiated by the addition of 1 ml PBS containing radiolabeled probe ([^{14}C]AG, [^{14}C]guanidine or [^{14}C]TEA) with or without inhibitors. Following incubation for a specified time period (as defined by the particular experiment), the buffer solution was aspirated and the monolayers quickly washed with three 1 ml aliquots of ice-cold PBS. Monolayers were solubilized with 0.5% Triton X-100 (1.2 ml) for a minimum of 1 h. A total of 1 ml of the solubilized monolayer was added to 10 ml of Scintiverse BD and counted in Packard Tri-Carb Liquid Scintillation Counter (Model 1900CA) with quench correction. Samples were normalized for protein content, as measured by the Lowry assay (Lowry et al., 1951). Nonspecific binding to the monolayer surface was determined by the addition of the radioactive probe in 1 ml of PBS, followed by immediate aspiration and rinsing. The time of incubation for nonspecific binding was typically < 5 s. Unless otherwise specified, all incubations were performed at room temperature.

The time course of intracellular accumulation of [^{14}C]AG was determined for both influx and efflux processes. Influx studies were initiated with the addition of [^{14}C]AG (10 or 200 μM, final concentration) in 1 ml PBS. Cultures (passages 44–50) were incubated for 1, 2.5, 5, 10, 15, 30, 60, 90, 120 or 150 min to establish the linear region of AG influx.

Efflux studies were conducted by preincubation of cultures (passage 98) with [^{14}C]AG (10 or 200 μM in 1 ml PBS) for 1 h. The radioactive solution was aspirated and the monolayers rinsed as previously described. Efflux of accumulated intracellular isotope was initiated by the addition of 1 ml of PBS. At 0, 1, 2.5, 5, 10, 15, 30, 60, 90 or 120 min the PBS was aspirated and the monolayers were solubilized for liquid scintillation counting of the intracellularly retained radioisotope.

2.3. Saturability experiments

Monolayers (at passage number of 63–67 or 79–82) were incubated for 1 min with increasing concentrations of AG (5, 10, 20, 50, 75, 100, 150, 200 and 300 μM, final concentrations). The specific activity of [^{14}C]AG ranged from 4.55 to 55.0 μCi/μmol.

Additional studies were performed to determine the temperature sensitivity of transport processes. Cultures (passage 81) were incubated at room temperature or at 0°C (on ice) with [^{14}C]AG (10 μM) with or without unlabelled A (1 mM) in room temperature or pre-chilled PBS, respectively.

2.4. Energy, ionic and voltage dependency

Several studies were performed to assess energy, ionic and voltage dependency of [^{14}C]AG cellular accumulation processes (passages 92–108). (1) [^{14}C]AG influx and efflux was assessed under conditions of reduced intracellular energy stores. Inhibition of metabolic cellular energy was accomplished by a 30 min preincubation with the metabolic poisons 2,4-dinitrophenol (250 μM final concentration) or sodium azide (NaN_3; 0.1%) in PBS. Control cultures were preincubated with PBS only. Following aspiration of the solutions and monolayer rinsing, cultures were incubated with [^{14}C]AG (10 μM) for either influx or efflux measurements. Influx was determined after a 5 min incubation period. Prior to the measurement of efflux, cultures were incubated with NaN_3 or PBS for 30 min plus another 30 min incubation with radioisotope followed by aspiration and monolayer washing. Efflux was measured after a 5 min incubation with PBS. (2) The dependence of [^{14}C]AG influx on sodium co-transport was tested by conducting experiments in control PBS buffer containing Na^+ or sodium-free buffer (components in millimolar concentration: KCl, 2.7; KH_2PO_4, 1.5; *N*-methylglucamine (NMG), 150; HCl, 150; pH 7.4) influx of [^{14}C]AG (10 μM) over a 5 min period was determined as previously described. (3) Hydrogen ion dependency was assessed as described by Yuan et al. (1991). Alteration of intracellular pH was confirmed using the fluorescent probe BCECF acetoxymethyl ester as described by Larsson et al. (1990) adapted for use with a fluorescent microplate reader (SpectraMAX Gemini, Molecular Devices, Sunnyvale, CA). In brief, three distinct experiments were performed. (a) Influx of [^{14}C] AG in the presence of an inwardly-directed proton gradient: influx was determined after a 5 min incubation of [^{14}C]AG (100 μM) in PBS, pH 7.4 or 5; (b) influx of [^{14}C]AG in the presence of an outwardly-directed proton gradient: monolayers were preincubated in PBS, pH 7.4 or 5, for 1 h, the media removed and the influx of [^{14}C]AG (10 μM) in PBS, pH 7.4 was determined after a 5 min incubation; and (c) efflux of [^{14}C]AG in the presence of an inwardly-directed proton gradient: monolayers were preincubated with [^{14}C]AG (100 μM) in PBS pH 7.4 for 30 min and efflux was determined after a 30 s incubation with PBS, pH 7.4 or 5. (4) The effect of membrane potential on [^{14}C]AG influx was assessed as described by Zevin et al. (1997) in (a) the presence of added valinomycin (1 μM final concentration) to create an outwardly-directed K^+ gradient and; (b) the presence of added valinomycin (1 μM) and K^+ (150 mM) to create a K^+ voltage clamp. Altered OK cell membrane potentials were successfully achieved by Loiseau et al. (1997) using similar methods. In brief, 10 μl of valinomycin stock solution (0.1 mM in ethanol) was added to 1 ml of PBS, and [^{14}C]AG (10 μM) influx was assessed after a 5 min incubation. Control cultures were incubated with 10 μl ethanol in PBS and [^{14}C]AG (10 μM). To create a potassium voltage clamp, cultures were incubated with valinomycin (1 μM) in Na^+-depleted/K^+ supplemented PBS buffer (components in millimolar concentrations: KCl, 141; K_2HPO_4, 9.5) and [^{14}C]AG (10 μM). Control cultures were incubated with PBS or in Na^+-depleted/NMG supplemented PBS buffer.

2.5. Substrate specificity experiments

A variety of influx and efflux experiments were performed to assess substrate specificity of the transport process. (1) Competitive influx studies were performed by the concurrent incubation of various inhibitors (1 mM) in combination with ^{14}C-AG (10 μM), [^{14}C]guanidine (20 μM) or [^{14}C]TEA (20 μM). Potential inhibitors tested were AG, TEA, guanidine, *N*-methylnicotinamide, cimetidine, procainamide, verapamil, quinidine sulfate, amiloride, clonidine, harmaline, di-aminoguanidine, methylguanidine, nitroguanidine, L-arginine and *p*-aminohippuric acid. Intracellular accumulation of radioisotope was determined after a 5 min incubation. (2) Influx counter transport studies were performed by preincubation with nonradiolabeled AG, guanidine or TEA (1 mM), for 30 min. Control monolayers were preincubated with PBS only. Intracellular accumulation of [^{14}C]AG was determined after a 5 min incubation with [^{14}C]AG (10 μM final concentration). (3) Efflux counter trans-

port studies were performed by preincubation of monolayers with [^{14}C]AG (10 μM final concentration) for 1 h, followed by aspiration and monolayer washing, as previously described. Unlabeled AG, guanidine or TEA (1 mM final concentration) was added to the cultures for a 5 min incubation. Control cultures were incubated with PBS only. The amount of intracellularly retained [^{14}C]AG was determined as described. Generally, OK cells at passage numbers 72–102 were used for the substrate specificity experiments involving [^{14}C]AG and [^{14}C]guanidine. Substrate specificity studies involving [^{14}C]TEA were performed at passages 64–66.

2.6. Data analysis

The specific activity of the radioisotope of interest was used to convert dpm to mass. Samples were normalized for protein content. The initial rate of influx or efflux was expressed as the mass of accumulated isotope/mg protein during the incubation period. For some experiments, data were expressed as a percentage of control influx or efflux rates.

Data were analyzed by an iterative nonlinear regression analysis (PCNONLIN, SCI Software; Lexington, KY) of the observed rate of AG influx versus AG concentration data. The data were modeled by one of two mathematical models (Eqns 1 or 2). The first model (Eq. (1)) describes only a passive diffusional component of [^{14}C]AG influx. Alternatively, the second model (Eq. (2)) describes a Michaelis-Menten saturable transport process function with an added concentration-dependent term to describe passive diffusion.

$$v_0 = k_{ns} * C \quad (1)$$

$$v_0 = \frac{V_{max} * C}{K_m + C} + (k_{ns} * C) \quad (2)$$

where v_o represents the initial rate of influx, V_{max} is the maximal rate of [^{14}C]AG influx, C represents drug concentration, K_m is the Michaelis-Menten equilibrium constant and k_{ns} is the influx rate constant attributable to diffusion. Parameter estimates of V_{max}, K_m and k_{ns} were obtained by iterative nonlinear regression analysis (PCNONLIN, SCI Software; Lexington, KY) of the observed rate of AG influx versus AG concentration data. Each data set was analyzed independently by each mathematical model. Selection of the model that best described the data was based on objective criteria that included Aikaike's information criterion, comparison of systematic deviations of fitted versus observed data and the sum of squared residuals.

All experiments were performed in triplicate on two or more different days. Statistical significance was determined by the unpaired Student's t-test or one-way ANOVA with post hoc testing, as specified. Data were statistically significant at $P <$ 0.05. Data are expressed as mean $\pm$ S.E.M. of (n) samples.

2.7. Materials

The OK cell line (CRL 1840) was purchased from the American Type Culture Collection (Rockville, MD). Culture media and supplements were supplied by Fisher Scientific (Pittsburgh, PA) and Intergen Corp. (Purchase, NY). Sterile tissue culture plasticware was supplied by VWR (Bridgeport, NJ). [^{14}C]Aminoguanidine (specific activity 55 mCi/mmol, purity $>98\%$) was procured from Moravek Biochemicals (Brea, CA). [^{14}C]Guanidine (specific activity 42 mCi/mmol, purity $>99\%$) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]Tetraethylammonium bromide (specific activity 56 mCi/mmol, purity $>98\%$) was purchased from Wizard Labs (Davies, CA). Scintiverse BD liquid scintillation cocktail was purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were supplied by Sigma Chemical (St. Louis, MO).

3. Results

3.1. Time course of influx and efflux

[^{14}C]AG accumulated in OK cell monolayers over a time period of 0–150 min. In separate preliminary studies, the initial linear influx rate occurred over the first 15 min and the initial linear

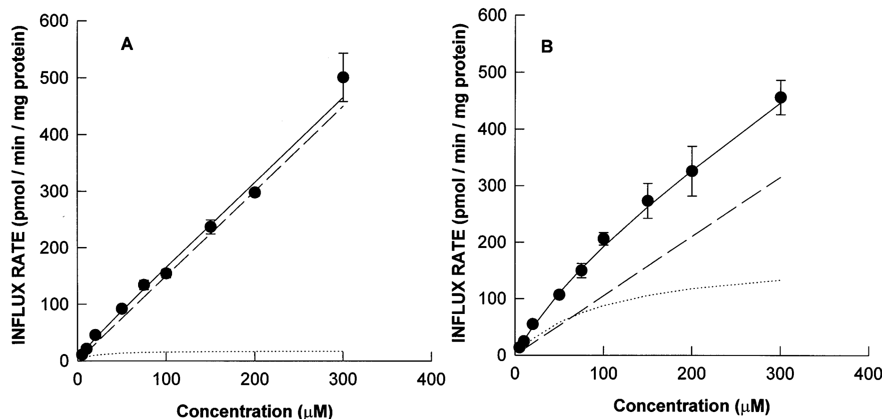

Fig. 1. Observed [14C]AG Influx rate versus AG concentration. The data were best described by saturable and diffusional processes (Eq. (2)). (A) Passage 63; and (B) passage 79. The solid line represents the predicted mathematical fit of the data. The dashed and dotted lines represent the mathematically resolved diffusional and saturable components, respectively. Data are mean ± S.E.M. ($n = 3$) of one representative experiment. The experiment was performed in triplicate.

efflux rate occurred over the first 10 min (data not shown). All subsequent transport experiments were performed during the initial linear rates, i.e. the first 5 min for both [14C]AG influx and efflux experiments.

3.2. Saturability of transport

Quite unexpectantly, the mechanism of AG influx differed as a function of passage number. Studies with higher passage numbers (> 79, Fig. 1B) resulted in an enhanced saturable [14C]AG influx process compared to studies conducted with lower passage numbers (< 67, Fig. 1A). The differences in observed saturable transport between lower and higher passage numbers were significant (passage #63–67; $V_{max} = 21.2 \pm 7.8$ pmol/(min*mg protein), $n = 3$ versus passage #79–82; $V_{max} = 129.7 \pm 24.3$ pmol/(min*mg protein), $n = 3$; $P < 0.05$; passage #63–67; $K_m = 23.7 \pm 10.8$ μM, $n = 3$ versus $K_m = 101.7 \pm 5.6$ μM, $n = 3$; $P < 0.05$). Nonsaturable influx (k_{ns}) did not vary with passage number (passage #63–67; $k_{ns} = 1.6 \pm 0.12$ pmol/(min*mg protein μM), $n = 3$ versus passage #79–82; $k_{ns} = 1.1 \pm 0.2$ pmol/(min*mg protein μM), $n = 3$; not significant (ns)).

The observed saturable transport process was temperature dependent (Fig. 2), consistent with a carrier mediated transport mechanism. [14C]AG influx was maximally decreased ($P < 0.05$) at 0°C, because inclusion of 1 mM AG in the incubation buffer did not further suppress influx, irrespective of temperature conditions.

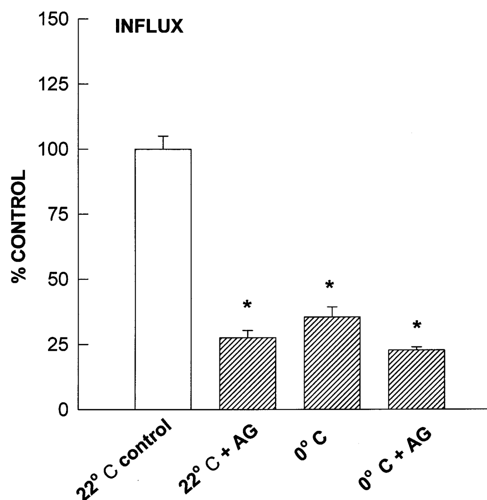

Fig. 2. Temperature effects on saturable [14C]AG influx. Maximal inhibition of [14C]AG influx was observed at 0°C, as well as with the addition of 1 mM AG at either 22 or 0°C. *$P < 0.05$ (compared with control) by one-way ANOVA with Student-Newman-Keuls' test. Data are mean ± S.E.M. ($n = 5$–6 wells) from two independent experiments (passage 81).

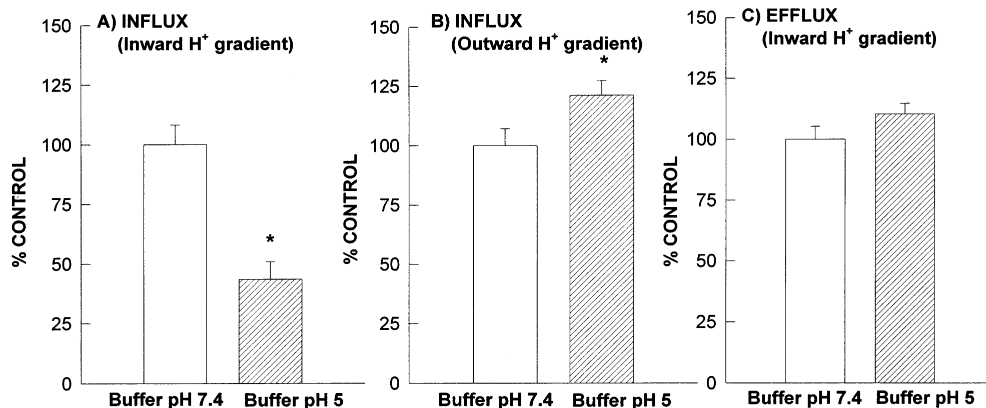

Fig. 3. The effect of proton gradients on [14C]AG influx. (A) Influx: inwardly directed proton gradient: [14C]AG was incubated in PBS of either pH 7.4 or 5. (B) Influx: outwardly directed proton gradient: to decrease intracellular pH, monolayers were preincubated with PBS of either pH 7.4 or 5. [14C]AG was incubated with PBS, pH 7.4. (C) Efflux: inwardly directed proton gradient: OK monolayers were preloaded with [14C]AG. Efflux of [14C]AG was determined in PBS of either pH 7.4 or 5. $*P < 0.05$ using unpaired Student's t-test. Data are mean ± S.E.M. ($n = 9$–12) of 3–4 independent experiments (passages 91–108).

3.3. Energy, ionic and voltage dependency-requirements

Several studies were performed to assess the dependency of [14C]AG transport on energy and ionic requirements. Exposure to the metabolic poisons, sodium azide and 2,4-dinitrophenol, did not significantly diminish [14C]AG influx (control: 27.8 ± 0.5, $n = 9$; NaN_3: 30.3 ± 3.1, $n = 9$; 2,4-dinitrophenol: 32.1 ± 1.4 pmol/mg protein, $n = 9$, ns). Similarly, [14C]AG efflux was not decreased by NaN_3 treatment (control: 144.8 ± 15.8, $n = 9$; NaN_3: 128.2 ± 11.3 pmol/mg protein, $n = 9$, ns). Replacement of Na^+ in the PBS buffer with NMG did not significantly decrease [14C]AG influx (Na^+-PBS: 25.2 ± 1.1, $n = 9$ versus NMG-PBS: 24.5 ± 1.6 pmol/mg protein, $n = 9$; ns). These data argue against a requirement for an ATP energy source or sodium gradient for [14C]AG saturable transport.

Fig. 3 shows the dependency of [14C]AG transport on proton gradients. The influx of [14C]AG was significantly reduced ($P < 0.05$) by PBS buffer, pH 5, compared to PBS buffer, pH 7.4 (Fig. 3A), most likely as a result of a competitive process. [14C]AG influx was significantly increased ($P < 0.05$) in the presence of an outwardly-directed proton gradient ($pH_{intracellular}$ 5; $pH_{extracellular}$ 7.4) (Fig. 3B). However, [14C]AG efflux was not significantly altered by incubation in PBS buffer, pH 5 (Fig. 3C). These data suggest that [14C]AG influx is dependent on proton gradients, that is, extracellular protons compete with extracellular AG for influx; intracellular protons are exchanged for extracellular AG.

[14C]AG influx was not altered by an outwardly-directed K^+ gradient (established by incubation in the presence of valinomycin; control: 28.8 ± 1.0, $n = 6$; versus with valinomycin: 28.5 ± 1.1 pmol/mg protein, $n = 6$; ns). Additionally, altering the membrane potential (i.e. voltage clamp conditions) by *iso*-osmotic substitution of sodium with potassium in the presence of valinomycin failed to modify [14C]AG influx (control buffer: 16.7 ± 0.4, $n = 6$; NMG buffer: 16.2 ± 1.4, $n = 6$; valinomycin + K^+ buffer: 16.4 ± 1.7 pmol/mg protein, $n = 9$; ns). These data imply that changes in membrane potential are unimportant in regulating [14C]AG influx.

3.4. Substrate specificity of transport

Competitive influx studies were performed utilizing various probes ([14C]AG, [14C]guanidine, [14C]TEA) and competitive inhibitors. Table 1 shows that compounds containing guanidino-

functional groups significantly inhibited [^{14}C]AG influx ($P < 0.05$), with two notable exceptions: L-arginine [a known substrate for cationic amino acid transporters: y^+ (Christensen and Antonioli, 1969), $b^{0,+}$ (Van Winkle et al., 1988), $B^{0,+}$ (Van Winkle et al., 1985) and y^+L (Devés et al., 1992)] and nitroguanidine, which is neutral at physiological pH as a result of the electronegative nitro group (Charton, 1965; Taylor and Wait, 1986). Similar results were observed for [^{14}C]guanidine (Table 1). Interestingly, AG, but not guanidine, significantly inhibited [^{14}C]TEA (Table 1). All of the tested organic cations (selected for specificity at the TEA transporter) significantly inhibited [^{14}C]AG, [^{14}C]guanidine or [^{14}C]TEA influx. Not unexpectedly, *p*-aminohippuric acid, a defined substrate of the organic anionic transporter (Foulkes and Miller, 1959), did not modulate [^{14}C]AG influx (Table 1). Influx counter transport studies revealed that AG, guanidine and TEA significantly ($P < 0.05$) *trans*-stimulated [^{14}C]AG influx (Fig. 4A). Corresponding efflux counter transport studies showed that AG and guanidine, but not TEA, significantly ($P < 0.05$) promoted *trans*-stimulation of [^{14}C]AG efflux (Fig. 4B).

4. Discussion

Collectively, these pharmacologic transport studies demonstrate that AG transport across cellular membranes involves both carrier-mediated and passive diffusion processes in the OK kidney cell line. Surprisingly, the apparent functional ac-

Table 1
Substrate specificity of the carrier-mediated process used by [^{14}C]AG, [^{14}C]guanidine or [^{14}C]TEA[a]

	[^{14}C]AG (10 μM)	[^{14}C]Guanidine (20 μM)	[^{14}C]TEA (20 μM)
	$n = 7$–9	$n = 8$–9	$n = 6$
I Guanidine analogues (1 mM)			
Control	100 ± 7.31	99.99 ± 4.12	99.99 ± 5.70
Aminoguanidine	45.66 ± 2.69*	31.06 ± 1.73*	68.97 ± 6.19*
Guanidine	45.94 ± 6.56*	44.49 ± 5.28*	85.06 ± 7.21
Methylguanidine	57.13 ± 5.24*	n.d.	n.d.
Diaminoguanidine	65.54 ± 5.78*	n.d.	n.d.
Nitroguanidine	121.07 ± 6.13	n.d.	n.d.
L-arginine	111.95 ± 10.93	143.44 ± 21.86	n.d.
II Organic cation transporter substrates/inhibitors (1 mM)			
Control	100 ± 7.31	99.99 ± 4.12	99.99 ± 5.70
Tetraethylammonium	69.31 ± 4.29*	45.44 ± 2.56*	70.21 ± 6.44*
N-methylnicotinamide	56.11 ± 4.72*	79.56 ± 5.04*	78.74 ± 5.42*
Cimetidine	51.68 ± 5.27*	79.72 ± 4.62*	59.23 ± 2.37*
Control[b]	100 ± 12.10	n.d.	n.d.
Procainamide	63.38 ± 4.02*	n.d.	n.d.
Verapamil	11.83 ± 3.32*	n.d.	n.d.
Quinidine sulfate	14.47 ± 2.07*	n.d.	n.d.
Amiloride	12.96 ± 2.44*	n.d.	n.d.
Clonidine	17.96 ± 2.27*	n.d.	n.d.
Harmaline	12.04 ± 1.53*	n.d.	n.d.
III Organic anion transporter substrate (1 mM)			
Control	99.98 ± 7.31	n.d.	n.d.
p-Aminohippuric acid	101.78 ± 7.73	n.d.	n.d.

[a] *$P < 0.05$ using one-way ANOVA and post hoc Dunnett's test. Data are represented as percent inhibition of control. Data represent mean ± S.E.M. ($n = 7$–12 wells from 3–4 independent experiments). n.d. = not done.
[b] [^{14}C]AG control for procainamide, verapamil, quinidine sulfate, amiloride, clonidine and harmaline.

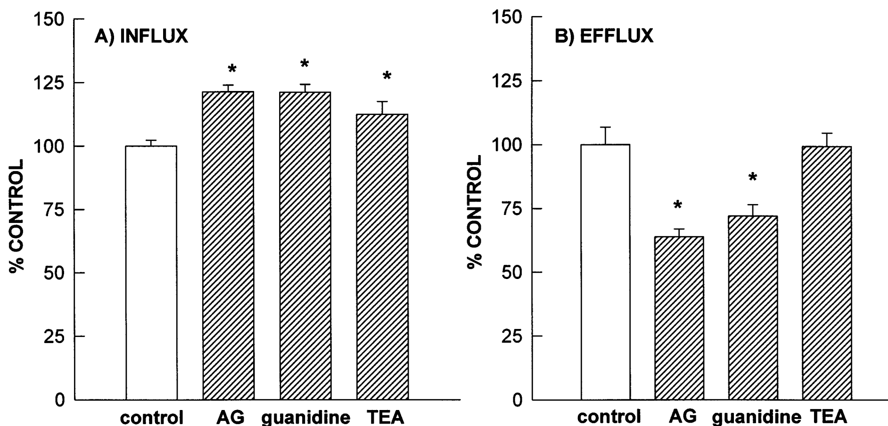

Fig. 4. *Trans*-stimulation of [^{14}C]AG by AG, guanidine or TEA. (A) OK cells were preloaded with 1 mM nonradiolabeled AG, guanidine or TEA, followed by the assessment of [^{14}C]AG Influx. (B) OK cells were preloaded with [^{14}C]AG, followed by incubation with AG, guanidine or TEA (1 mM in PBS, pH 7.4). $*P < 0.05$ by one-way ANOVA with Dunnett's test. Data are mean ± S.E.M. ($n = 6$–9) of 2–3 independent experiments (passage 99–102).

tivity of the carrier mediated transporter increased with passage number, as demonstrated by the observed increase in saturable AG transport in passage numbers > 79. This observation can be interpreted as a novel *gain of function* with successive passaging of cultures. The increased V_{max} and K_m parameters at higher passage numbers could reflect increased expression of a transporter(s) mediating substrate influx.

However, it is more commonly reported that cell cultures typically show a loss of function with increased passage number, such as a loss of alkaline phosphatase activity and functional expression of brush border enzymes and transport proteins in Caco-2 cells (Briske-Anderson et al., 1997; Yu et al., 1997). Thus an alternative interpretation is a *loss of function* with successive passages, reflective of a decreased expression of a distinct transporter(s) mediating aminoguanidine/guanidine efflux. To date, there are no documented studies defining aminoguanidine/-guanidine transport-mediated efflux in any system (in vitro or in vivo). It can be hypothesized that aminoguanidine and guanidine could be effluxed by P-glycoprotein (P-gp), given that these compounds are weak organic bases. In preliminary studies using the 9L glioblastoma cell line (which expresses P-gp, Yamashima et al., 1993), neither aminoguanidine nor guanidine evoked significant intracellular accumulation of rhodamine 123, in contrast to that observed with cyclosporin A, verapamil and quinidine (Kuo and Boje, personal communication). These findings suggest that neither aminoguanidine nor guanidine are substrates for P-gp, and that if efflux does occur, it occurs by a unique, presently unknown transport protein.

Evidence for a saturable, carrier-mediated process for AG transport is summarized as follows: (1) observed AG transport is saturable, temperature dependent and bi-directional. (2) The observed transport process does not require ATP or sodium, and does not rely on membrane potential as a driving force. (3) Observed AG influx is enhanced by AG, TEA, guanidine and protons at the *trans* side of the membrane. (4) Observed AG efflux is also *trans*-stimulated by AG and guanidine, but not by TEA or protons. (5) The observed transport process has a broad substrate specificity that encompasses both well known substrates of the organic cation transporter (Holohan and Ross, 1981; Inui et al., 1985; McKinney and Kunnemann, 1985; Sokol et al., 1985; Rafizadeh et al., 1986; McKinney and Kunnemann, 1987; Miyamoto et al., 1989) as well as compounds with guanidino functional groups.

One interesting finding was that TEA and protons independently *trans*-stimulated [^{14}C]AG

influx, but failed to correspondingly *trans*-stimulate [^{14}C]AG efflux. These contrasting findings of *trans*-stimulation could be possibly explained by an asymmetry of the translocation rate of the carrier protein-substrate complex. Asymmetry of translocation rates is a phenomenon common to many transport proteins (Basketter and Widdas, 1978; Devés and Krupka, 1978), and can arise as a result of differences in unbound free energy requirements of transport protein localization or conformation at the inner versus outer membrane faces (Stein and Honig, 1977). Moreover, translocation rate asymmetry was reported to occur with the organic cations mepiperphenidol and TEA in renal brush border vesicular membranes of dog kidney cortex (Holohan and Ross, 1980). One can speculate that the transport protein for [^{14}C]AG exists predominately in a particular conformation at the inner membrane face, thereby possibly explaining the TEA and proton *trans*-stimulation of ^{14}C-AG influx and lack of corresponding effects on [^{14}C]AG efflux.

The results of the present study identify a broad substrate specificity of the transporter(s) that mediate AG saturable influx. [^{14}C]AG and [^{14}C]guanidine influx were inhibited by known substrates of the guanidine transporter and organic cationic transporter, with no apparent difference in the extent of inhibition by the various substrates. Similarly, there were no observable differences in the inhibition of [^{14}C]TEA influx by known substrates of the guanidine or OCT transporters. Based on the data, it is clear that [^{14}C]AG does not utilize the specific transporters for basic amino acids (L-arginine) (Christensen and Antonioli, 1969; Van Winkle et al., 1985, 1988; Devés et al., 1992) or organic anions (*p*-aminohippuric acid) (Foulkes and Miller, 1959).

The literature describes two distinct types of renal tubule transporters at the brush border membrane: an organic cation transporter and a guanidine transporter (Miyamoto et al., 1989; Chun et al., 1997). The pharmacological characteristics of the organic cation/proton exchange transporter is reasonably well defined in the OK cell line (Yuan et al., 1991). However, the existence and characteristics of a guanidine transporter in OK cultures is not known. The situation is further complicated in that there may be apparent species and tissue differences with respect to the guanidine transporter (Chun et al., 1997; Zevin et al., 1997; Zhang et al., 1997a).

The findings of the present study do not clearly discriminate whether [^{14}C]AG transport is mediated exclusively by the organic cationic transporter or guanidine transporter. Consistent with the findings of this study, both TEA and guanidine were reported to inhibit substrate influx of the organic cationic and guanidine transporter in a human choriocarcinoma cell line (Zevin et al., 1997). In contrast, both TEA and *N*-methylnicotinamide failed to inhibit guanidine transport in renal brush border membrane vesicles (Miyamoto et al., 1989; Chun et al., 1997). Both types of transporters demonstrate *trans*-stimulation or counterflux phenomenon (Yuan et al., 1991; Chun et al., 1997), as was observed in the present study. Similarly, outwardly-directed proton gradients enhance substrate influx for both transporters (Inui et al., 1985; Miyamoto et al., 1989; Yuan et al., 1991; Chun et al., 1997). An unequivocal identification of the transporter(s) involved in AG transport requires molecular biological approaches.

Fig. 5 summarizes the general findings of the present study. In summary, AG saturable transport is temperature dependent; independent of ATP, Na^{+} or membrane potential; dependent on proton gradients; and exhibits *trans*-stimulation of transport processes.

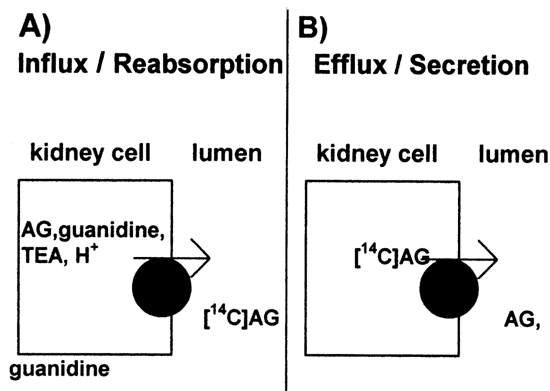

Fig. 5. Summary of aminoguanidine transport across the apical membranes of opossum kidney cell cultures. (A) Influx; and (B) efflux.

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