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# Modeling of Cellular Arginine Uptake by More Than One Transporter

**Marietha J. Nel · Angela J. Woodiwiss · Geoffrey P. Candy**

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**Abstract** Determining the kinetic constants of arginine uptake by endothelial cells mediated by more than one transporter from linearization of data as Eadie-Hofstee plots or modeling which does not include the concentration of trace radiolabeled amino acid used to measure uptake may not be correct. The initial rate of uptake of trace [$^3$H]L-arginine by HUVECs and $ECV_{304}$ cells in the presence of a range of unlabeled arginine and modifiers was used in nonlinear models to calculate the constants of arginine uptake using GraphPad Prism. Theoretical plots of uptake derived from constants determined from Eadie-Hofstee graphs overestimated uptake, whereas those from the nonlinear modeling approach agreed with experimental data. The contribution of uptake by individual transporters could be modeled and showed that leucine inhibited the individual transporters differently and not necessarily competitively. *N*-Ethylmaleimide inhibited only $y^+$ transport, and BCH may be a selective inhibitor of $y^+L$ transport. The absence of sodium reduced arginine uptake by $y^+L$ transport and reduced the $K_m'$, whereas reducing sodium decreased arginine uptake by $y^+$ transport without affecting the $K_m'$. The nonlinear modeling approach using raw data avoided the errors inherent in methods deriving constants from the linearization of the uptake processes following Michaelian kinetics. This study provides explanations for discrepancies in the literature and suggests that a nonlinear modeling approach better characterizes the kinetics of amino acid uptake into cells by more than one transporter.

**Keywords** Amino acid transport · Kinetics · Endothelial cell

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## Introduction

The semi-essential, cationic amino acid L-arginine plays an important role in cellular function and metabolism and is the known precursor of the vasodilator nitric oxide (NO). Arginine potentiates the release of NO from endothelial cells grown in arginine-depleted medium, thereby regulating NO production (Sessa et al. 1990). Arginine may limit NO production in subjects with normal blood pressure but not in patients with hypertension (Panza et al. 1993). Indeed, fasting plasma arginine concentrations are elevated in patients with hypertension (Penttinen et al. 2000; Moss et al. 2004; Perticone et al. 2005; Naidoo et al. 2009), yet supplemental arginine can decrease blood pressure in such patients (Siani et al. 2000; Ast et al. 2010). One explanation for these data may be that arginine uptake into cells was impaired and therefore would not be available for NO production. In support of such a hypothesis, studies have shown that cellular uptake of arginine is reduced in lymphocytes from patients with hypertension and individuals genetically predisposed to developing hypertension (Schlaich et al. 2004). However, elucidating the kinetics of arginine uptake into endothelial cells is fundamental to determining whether arginine uptake is indeed impaired in these cases.

Studies to date have determined that cationic amino acid uptake into endothelial cells is mediated by the high-

affinity/low-rate $y^+L$ transporter and the low-affinity/high-rate $y^+$ transporter. Uptake mediated by the $y^+$ transporter is sodium-independent, pH-insensitive, stereoselective and inhibited by *N*-ethylmaleimide (NEM) and certain neutral amino acids (Devés and Boyd 1998). Transport of arginine and other cationic amino acids by the $y^+L$ transporter is sodium-independent; and in the presence of sodium, neutral amino acids, such as methionine, glutamine and leucine, may also be transported with high affinity (Devés and Boyd 1998; Mann et al. 2003; Bröer 2008). Although recent studies have described the activity and properties of transporter gene products CAT-1, CAT-2A and CAT-2B ($y^+$) as well as 4F2hc/$y^+$LAT1 (SLC3A2/SLC7A7) and 4F2hc/$y^+$LAT2 (SLC3A2/SLC7A6) ($y^+L$) (Closs et al. 1997; Mann et al. 2003; Bröer 2008), ascribing and reconciling the activities of these gene products to earlier studies which only measured activity can be difficult as the use of different cell types and experimental approaches when determining uptake rates has complicated direct comparison of the data.

Most studies determining the uptake of radiolabeled amino acids have assumed Michaelis–Menten kinetics and have calculated constants from Lineweaver–Burk reciprocal plots, Eadie-Hofstee plots and nonlinear modeling (Christensen and Antonioli 1969; White and Christensen 1982; Closs et al. 1993b; Moss et al. 2004; Brunini et al. 2006). Determining the kinetics, the relative contribution and the physiological importance of individual transporters in cells expressing more than one transporter may be complicated (Rotmann et al. 2007). Indeed, early in vitro kinetic uptake studies that ascribed uptake to only $y^+$ transport (Christensen et al. 1994) appear to have overlooked the contribution of $y^+L$ transport (Devés et al. 1992). Furthermore, when determining kinetic parameters of individual cationic amino acid transporters operating simultaneously in a cell, studies have made various assumptions.

1. Studies have measured uptake of trace-labeled substrate in the presence of unlabeled substrate, without factoring in the relative concentrations of these when calculating the kinetic parameters (Sobrevia et al. 1995; Durante et al. 1996; Dall'Asta et al. 2000; Casanello and Sobrevia 2002; Hardy and May 2002; Arancibia-Garavilla et al. 2003). In this regard, the rate of uptake of labeled amino acid in the presence of unlabeled amino acid is not linear (Devés et al. 1992).
2. Studies in which radiolabeled tracers have been used with subtraction of nonspecific uptake and then Eadie-Hofstee linearization of data are not suitable to determine the existence of more than one transporter of uptake (Malo and Berteloot 1991).
3. Studies determining relative uptake rates at specific concentrations of the amino acids and their inhibitors (Arancibia-Garavilla et al. 2003; Signorello et al. 2003; Rotoli et al. 2005) may not infer that such rates apply at other substrate and/or inhibitor concentrations.
4. Studies have measured total uptake, then inhibited one of the transporters to determine the residual activity of the other transporter by subtraction. This assumes a linear and additive uptake independent of trace-labeled and unlabeled substrate concentrations (Mendes-Ribeiro et al. 1999; Ayuk et al. 2002; Brunini et al. 2006; Rotmann et al. 2007).
5. Studies have calculated kinetic constants from models of competitive inhibition of cationic amino acid uptake by structurally dissimilar neutral amino acids, such as leucine and glutamine (Devés et al. 1992, 1993), without determining whether noncompetitive or other forms of inhibition significantly improved the fit with the experimental data.
6. Studies determining inhibitor concentrations at which uptake was inhibited by 50% ($I_{50}$) (Hardy and May 2002; Rotoli et al. 2005) have also only modeled competitive inhibition (Cheng and Prusoff 1973) by these structurally dissimilar neutral amino acids (Angelo et al. 2005; Dall'Asta et al. 2000).

To address these issues, we modeled cationic amino acid uptake into cells using the general nonlinear approach of Malo and Berteloot (1991). This approach allows initial rates of uptake by more than one transporter to be determined and importantly includes the actual concentrations of both the trace-radiolabeled and unlabeled amino acid in the model. Furthermore, no assumptions are made regarding the type of inhibition and the concentrations of inhibitors (or activators) included in the model. As the model was additive, the theoretical contribution of uptake by each transporter could be modeled.

We tested this approach by modeling the initial rate kinetics of arginine uptake by two transporters into $ECV_{304}$ cells, over a range of substrate and inhibitor concentrations, and reproduced the approach in primary human umbilical cord vein endothelial cells (HUVECs). Importantly, the results explain discrepancies in the literature when determining the kinetic parameters of cationic amino acid transport by more than one transporter.

## Materials and Methods

### Ethical Approval

Although the study did not involve patients, the Human Research Ethics Committee of the University of the Witwatersrand approved it (approval: M03-09-35, October 16, 2003).

## Materials

$ECV_{304}$ CRL-1998 and primary HUVECs were obtained from the American Type Culture Collection (Rockville, MD) and Lonza (Walkersville, MD), respectively. Fetal calf serum (FCS) (GIBCO, Carlsbad, CA) and medium 199 (M199) culture medium were obtained in powder form (Highveld Biologicals, Sandringham, Johannesburg, South Africa). The latter was dissolved in double-glass distilled water and filtered through 0.22-μm Millipore filters in a stainless steel filter apparatus (Millipore, Billerica, MA). Costar disposable plastic cell cultureware (Corning, Pittsburgh, PA) was used for all cell culture experiments. Phosphate-buffered saline (PBS), NEM and 2-aminobicyclo[2,2,1] heptane-2-carboxylic acid (BCH) were obtained from Sigma-Aldrich (St. Louis, MO). [$^3$H]L-Arginine was obtained from New England Nuclear and Ultima Gold scintillation fluid from Perkin Elmer (Boston, MA).

## Cell Culture and Arginine Uptake Assay

For all experiments, HUVECs or $ECV_{304}$ cells with contact inhibition characteristics were grown to confluence as an adherent monolayer at 37°C in 5% $CO_2$ according to the suppliers' instructions. Cells were plated out ($4 \times 10^5$/well) in six-well culture plates with 2 ml complete M199 medium supplemented with 10% FCS (Gazzola et al. 1981) and incubated for 24 h. The medium was removed, cells were washed once in 1 ml PBS (pH 7.4) and 2 ml M199 without FCS, glutamine or arginine was added to deplete the cells of arginine and cells were incubated for a further 24 h as previously described (Sessa et al. 1990). Cell viability was determined by exclusion of trypan blue dye. Cells were then washed twice in 1 ml PBS, and PBS containing freshly diluted labeled 10 nM [$^3$H]L-arginine, with a range of concentrations of unlabeled arginine with or without inhibitors, such as leucine, was added. Uptake was measured for 30 s (Gazzola et al. 1981; White and Christensen 1982; Sala et al. 2002) while agitating the culture plate. The test solution was removed, and the cells were rapidly washed twice with cold PBS before 10% trichloroacetic acid in deionized water was added for 30 min at room temperature, to stop the uptake (Gazzola et al. 1981). Cells were then scraped off the culture plate and resuspended in 800 μl of distilled water. Following this, 200 μl of the cell suspension was added to 4 ml of scintillation fluid and the radioactivity counted (1600CA Tricarb; Canberra Packard, Meriden, IL).

To determine the effect of sodium on arginine uptake, uptake was measured using the following three buffers: (1) Krebs buffer (157 mM sodium), (2) Krebs buffer in which the sodium chloride was replaced with choline chloride (26 mM sodium) and (3) sodium-free buffer in which the other sodium-containing salts in the Krebs buffer were replaced with potassium (Christensen and Antonioli 1969; Mendes-Ribeiro et al. 1999; Arancibia-Garavilla et al. 2003). The effects of the sulfhydryl inhibitor NEM and the neutral amino acid analogue BCH on arginine uptake were determined by preincubating cells with these inhibitors prior to the determination of arginine uptake.

## Determination of Uptake Kinetic Constants

In this study, it was assumed that the uptake of $^3$H-labeled and that of unlabeled arginine were identical. In preliminary experiments we determined the linearity of labeled arginine uptake with time and concentration. Further, uptake of labeled arginine in the presence of unlabeled arginine with time was determined. Uptake of radiolabeled arginine by the cells was expressed as nanomoles per $4 \times 10^5$ cells per minute, and these data were used directly in nonlinear models to determine the kinetic constants using methods described previously (Malo and Berteloot 1991).

The rate of uptake ($v_T$) of undiluted tracer ($T$) by a single transporter, in the presence of unlabeled substrate ($S$), may be described by Michaelis–Menten kinetics (Malo and Berteloot 1991):

$$v_T = \frac{V_{\max} * [T]}{(K_m + [T] + [S])} \quad (1)$$

If two independent transporters (denoted by the subscripts "a" and "b") were present, in the presence of unlabeled substrate and diffusion ($K_D * [T]$), the rate of uptake of the labeled substrate was additive:

$$v_T = \frac{V_{maxa} * [T]}{(K_{ma} + [T] + [S])} + \frac{V_{maxb} * [T]}{(K_{mb} + [T] + [S])} + K_D * [T] \quad (2)$$

After correcting for dilution of the isotope, rate versus substrate plots were constructed, assuming an additive model for two transporters following Michaelis–Menten kinetics, and the data were linearized as Eadie-Hofstee plots to determine the kinetic constants, which, if curvilinear, may suggest two independent transporters (Malo and Berteloot 1991).

## Statistical Analysis

Equations 1 and 2 were used to model the uptake of the 10 nM labeled arginine in the presence of a range of concentrations of unlabeled arginine and various inhibitors. The maximal rates of uptake ($V_{maxa}$, $V_{maxb}$) and the Michaelis constants ($K_{ma}$, $K_{mb}$) were adjusted using nonlinear regression analysis (GraphPad Prism®, version 5; GraphPad Software, La Jolla, CA) to best fit the model to

the experimental data. As the rate of uptake was fastest at low substrate concentrations and slowest at high substrate concentrations, the curve was fitted by minimizing the sum of squares of relative distances ($1/Y^2$ weighting to restore equal weighting to all points in the curve) of the data from the curve. Further, after checking for outliers, a D'Agostino and Pearson normality test ($P > 0.20$) was used to ensure that residuals were randomly distributed across the substrate concentrations. A robust fit of data which is less sensitive to outliers was also used. $V_{maxa}$, $V_{maxb}$, $K_{ma}$ and $K_{mb}$ were reported as mean ± SEM or mean ± SD, as indicated.

Models of one- versus two-transporter uptake were tested, and it was determined whether the inclusion of a diffusion component improved the fit (Eq. 2). In the presence of inhibitors, the type of inhibition was elucidated by determining whether either $V_{max}'$ and/or $K_m'$ and the ratio $K_m'/V_{max}'$ remained constant. The inhibition constants ($K_i$) were then calculated for both transporters (Krupyanko 2007). As the results were in agreement with the literature, transport by the higher-affinity/lower-rate transporter (denoted by subscript "a" when $K_{ma}$, $V_{maxa}$ and $K_{ia}$ were reported) was also referred to as the $y^+L$ transporter. Similarly, the lower-affinity/faster-rate transporter (denoted by subscript "b" when $K_{mb}$, $V_{maxb}$ and $K_{ib}$ were reported) was referred to as the $y^+$ transporter.

## Results

### Preliminary Results

Arginine uptake into $ECV_{304}$ cells was linear up to 50 nM $^3$H-arginine (Fig. 1a) and for up to 5 min (Fig. 1b). Initial experiments found that 30 s was the shortest time when the rate could be reproducibly measured under the experimental conditions used (data not shown). In the presence of unlabeled arginine, the rate of uptake of 10 nM $^3$H-labeled arginine decreased in an exponential manner over 5 min and depended on the concentration of unlabeled arginine (Fig. 1c). At 30 s, the nonlinear dependence of the rate of 10 nM $^3$H-arginine uptake upon the concentration of unlabeled arginine was shown for both $ECV_{304}$ cells (Fig. 2a) and HUVECs (Fig. 3a).

### Arginine Uptake by $ECV_{304}$ Cells and HUVECs

Kinetic constants, derived from dilution-corrected, linear-transformed plot intercepts, were used as preliminary estimates in the nonlinear additive models of uptake (Malo and Berteloot 1991). For both $ECV_{304}$ cells (Fig. 2a) and HUVECs (Fig. 3a), after excluding outliers and ensuring residuals between experimental data and the model fit were normally distributed within the range of unlabeled arginine

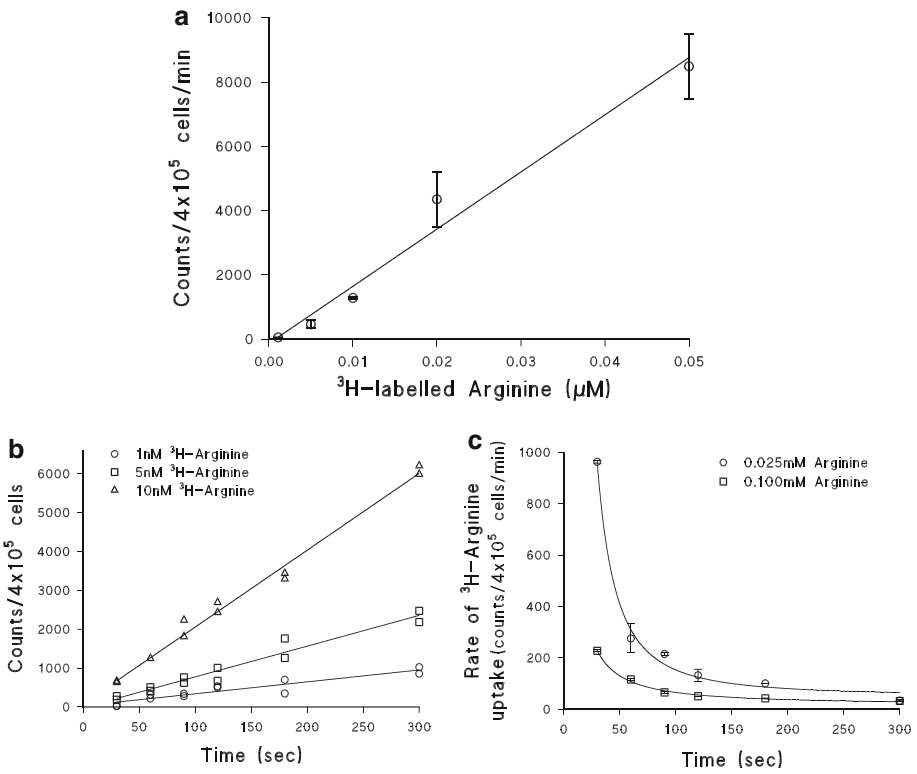


**Fig. 1** Uptake of labeled arginine by $ECV_{304}$ cells. **a** Uptake with concentration of $^3$H-arginine (mean ± SD from triplicate experiments). **b** Uptake over time with concentration of $^3$H-arginine. **c** Nonlinear decrease in the rate of 10 nM $^3$H-arginine uptake in the presence of 0.025 and 0.100 mM unlabeled arginine with time. Experimental details are described in Materials and Methods except that uptake was measured for up to 300 s before the reaction was stopped. Data for HUVECs were similar (data not shown)

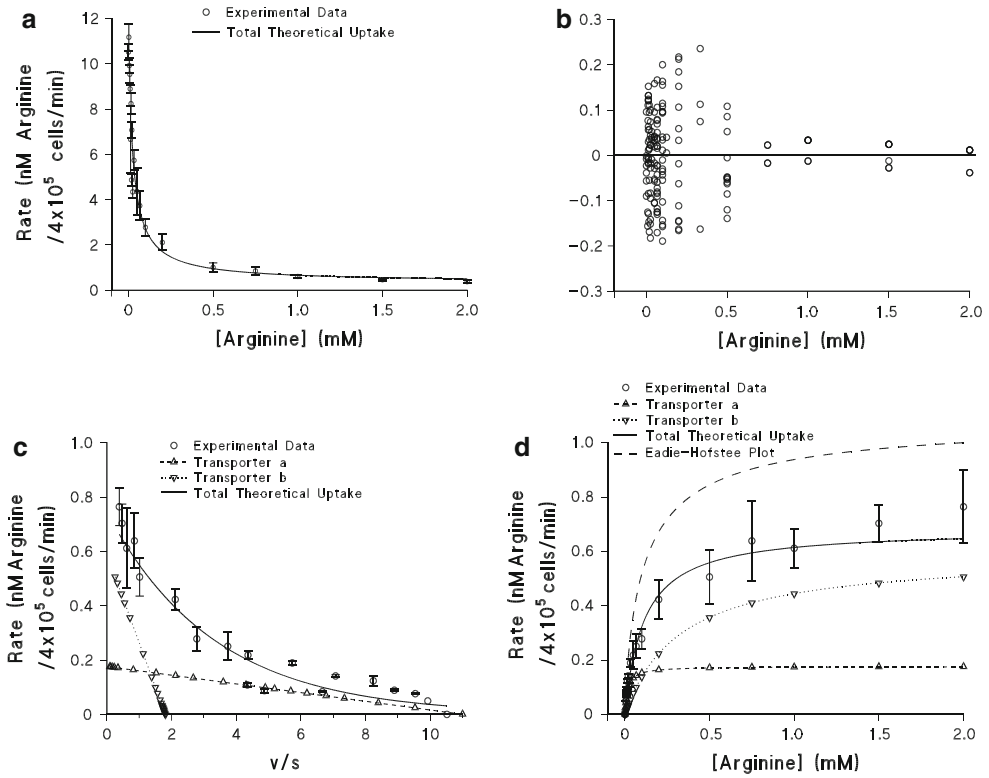


**Fig. 2** Data used to determine the kinetic constants of arginine uptake by nonlinear modeling for $ECV_{304}$ cells. **a** Experimental uptake (mean ± SD from 10 experiments) of 10 nM $^3$H-arginine in the presence of increasing concentrations of unlabeled arginine was used with Eq. 2 to determine the kinetic constants (see Materials and Methods). The curve is the theoretical uptake derived from these constants (see Table 1). **b** Residuals for graph **a**. A D'Agostino and Pearson omnibus K2, $P = 0.383$, indicated a lack of bias for the differences between experimental arginine uptake and the theoretical model contributions. **c** Eadie-Hofstee plots calculated from dilution-corrected data from **a** showing close agreement between total experimental uptake (mean ± SD) and total theoretical uptake calculated from the derived constants of the nonlinear modeling in Table 1. *Straight lines* are the theoretical plots (derived from the constants of nonlinear modeling in Table 1) for each transporter to illustrate the differences between these lines and the theoretical curve of total transport. **d** Michaelis–Menten graphs of dilution-corrected experimental rate of arginine uptake (as mean ± SD) and theoretical contributions of transporters "a" ($y^+L$, *dotted line*) and "b" ($y^+$, *short dashed line*) and the combined total theoretical uptake (*solid line*) (derived from the constants obtained by nonlinear modeling, Table 1), which agreed well with the experimental data. Also shown is the curve (*long dashed line*) calculated from the constants derived from the Eadie-Hofstee plots of dilution-corrected data (Table 1), which overestimated the experimental uptake. Experimental details are described in Materials and Methods with uptake measured for 30 s

tested (Figs. 2b, 3b), a model of uptake by two transporters was preferred over that of a single transporter, irrespective of whether the models included a diffusion component ($P < 0.0001$, data not shown). The two-transporter model was also preferred to a single-transporter model with $n$ substrate binding sites and with diffusion (see equation 3 in Chenu and Berteloot 1993; data not shown). Furthermore, the Eadie-Hofstee plots for both $ECV_{304}$ cells (Fig. 2c) and HUVECs (Fig. 3c) were curvilinear, hence supporting a model of uptake by two transporters.

In both $ECV_{304}$ cells and HUVECs, the kinetics program indicated that an uptake model of two transporters without diffusion was preferred, although the difference between the model with and that without diffusion was not significant (Table 1). For both cell types, models including diffusion were found to have large errors (SEM) for the estimates of the kinetic constants ($K_{mb}$) and the diffusion constant ($K_D$), with wide confidence intervals for the diffusion constant (Table 1), supporting the indication that the model without diffusion was correct. Furthermore, a robust fit (less sensitive to outliers) of the data found the diffusion parameter was different from that calculated by the least squares fit method, further indicating that the model including diffusion was not stable when fitting this model to the data. Finally, the contribution by diffusion of 10 nM $^3$H-arginine used in these experiments to the total uptake was small ($K_D$ * 0.00001 mM, Eq. 2). Therefore, for subsequent experiments the diffusion component was excluded.

The $V_{maxa}$ ($y^+L$ transport) calculated for $ECV_{304}$ cells was similar to that of the HUVECs ($P = 0.34$), whereas the $V_{maxb}$ ($y^+$ transport) was higher for HUVECs compared to $ECV_{304}$ cells ($P = 0.019$). $ECV_{304}$ cell affinity constants for both transporters were similar to those for HUVECs

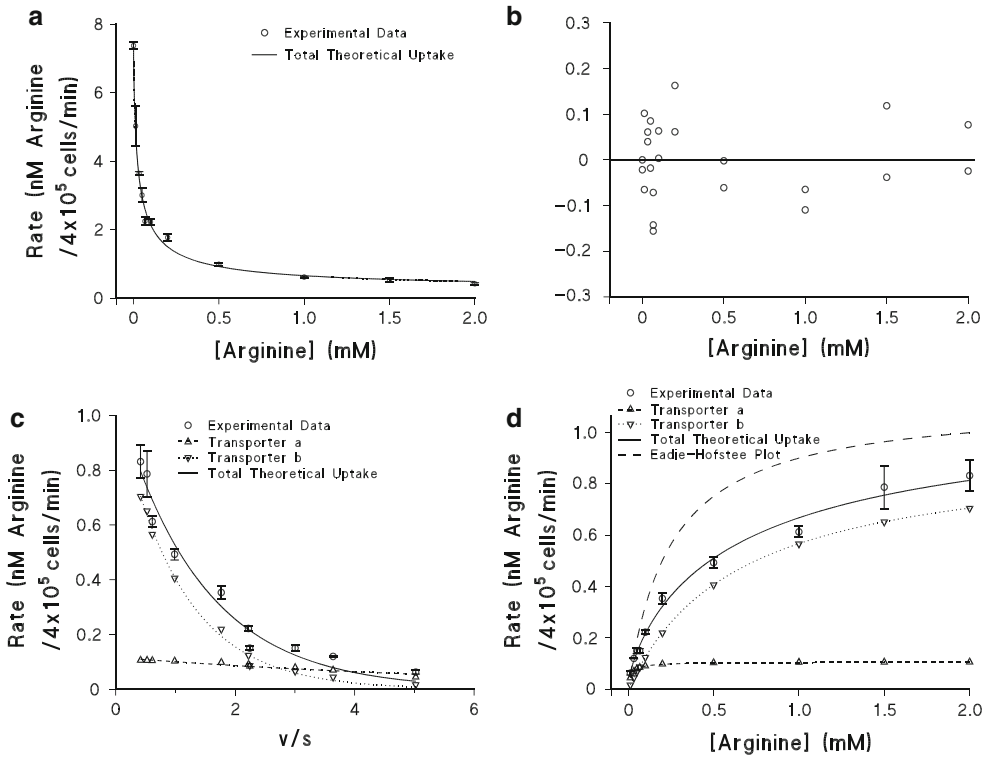


**Fig. 3** As for Fig. 2 showing the data used to determine the kinetic constants of arginine uptake by nonlinear modeling for HUVECs. **a** Experimental uptake (mean ± SD from duplicate experiments) of 10 nM $^3$H-arginine in the presence of increasing concentrations of unlabeled arginine was used with Eq. 2 to determine the kinetic constants (see Materials and Methods). **b** Residuals for graph **a**. A D'Agostino and Pearson omnibus K2, $P = 0.858$, indicated a lack of bias for the differences between experimental arginine uptake and the theoretical model contributions. **c** Eadie-Hofstee plots calculated from dilution-corrected data from **a** showing close agreement between total experimental uptake (mean ± SD) and total theoretical uptake calculated from the derived constants of the nonlinear modeling in Table 1. *Straight lines* are the theoretical plots (derived from the constants of nonlinear modeling in Table 1) for each transporter to illustrate the differences between these lines and the theoretical curve of total transport. **d** Michaelis–Menten graphs of dilution-corrected experimental rate of arginine uptake (mean ± SD) and theoretical contributions of transporters "a" ($y^+$L, *dotted line*) and "b" ($y^+$, *short dashed line*) and the combined total theoretical uptake (*solid line*) (derived from the constants obtained by nonlinear modeling, Table 1), which agreed well with the experimental data. Also shown is the curve (*long dashed line*) calculated from the constants derived from the Eadie-Hofstee plots of dilution-corrected data (Table 1), which overestimated the experimental uptake. Experimental details are described in Materials and Methods with uptake measured for 30 s

($K_{ma}$ and $K_{mb}$; Table 1; Figs. 2d, 3d). The relative maximum uptake ($V_{maxb}/V_{maxa}$) of the faster/lower-affinity transporter ($y^+$) relative to the slower/higher-affinity ($y^+$L) transporter was 3.6 in $ECV_{304}$ cells and 8.8 in HUVECs. The kinetic parameters determined by the nonlinear modeling approach were similar to those determined using a model of Michaelis–Menten uptake with two transporters, after correcting uptake for dilution of the isotope (Figs. 2d, 3d).

The additive model approach allowed for the theoretical contribution of uptake by the individual transporters into $ECV_{304}$ cells (Fig. 2d) and HUVECs (Fig. 3d). Theoretical total uptake calculated from the kinetic constants, derived from dilution-corrected, linear-transformed plot intercepts (Eadie-Hofstee plots, Table 1), significantly overestimated the experimental data, whereas theoretical uptake calculated from nonlinear modeling closely matched the experimental data (Table 1; Fig. 2d for $ECV_{304}$ cells and Fig. 3d for HUVECs). For both cell lines, the $y^+$L transporter (transporter a) was the major contributor of uptake at low arginine concentrations and approached $V_{maxa}$ above 0.300 mM with near zero order kinetics above this concentration. The $y^+$ transporter (transporter b) accounted for most of the transport above this concentration.

Finally, we recalculated the kinetic constants using dilution-corrected data, modeling an additive Michaelis–Menten equation to best fit the experimental data, to compare the results obtained with equation 2 of the nonlinear modeling using the raw data as described above. From the Michaelis–Menten graph (Fig. 2d), the constants determined for $ECV_{304}$ cells were $V_{maxa} = 0.176 \pm 0.027$ nM arginine/$4 \times 10^5$ cells/min, $K_{ma} = 0.016 \pm 0.002$ mM, $V_{maxb} = 0.589 \pm 0.028$ nM arginine/$4 \times 10^5$ cells/min and $K_{mb} = 0.326 \pm 0.072$ mM, which were similar to those obtained from the nonlinear modeling (i.e., the kinetic constants obtained from the model fit of Fig. 2d matched those of the fit of Fig. 2a; Table 1). Similar

**Table 1** Comparison of kinetic constants obtained from Eadie-Hofstee plots (from dilution corrected data) and nonlinear models (using raw data) of arginine uptake by $ECV_{304}$ cells and HUVECs

| | $ECV_{304}$ cells | | | HUVECs | | |
|---|---|---|---|---|---|---|
| | Eadie-Hofstee plot | Model without diffusion[a] | Model with diffusion | Eadie-Hofstee plot | Model without diffusion[b] | Model with diffusion |
| $V_{maxa}$ (nM arginine/ $4 \times 10^5$ cells/min) | 0.294 ± 0.042* | 0.176 ± 0.026 (0.243) | 0.136 ± 0.034 (0.235) | 0.225 ± 0.019** | 0.106 ± 0.026 (0.122) | 0.091 ± 0.037 (0.120) |
| $K_{ma}$ (mM) | 0.024 ± 0.006 | 0.016 ± 0.002 (0.021) | 0.013 ± 0.003 (0.021) | 0.030 ± 0.005 | 0.018 ± 0.004 (0.020) | 0.016 ± 0.005 (0.020) |
| $V_{maxb}$ (nM arginine/ $4 \times 10^5$ cells/min) | 0.768 ± 0.035*** | 0.589 ± 0.028 (0.558) | 0.459 ± 0.054 (0.407) | 1.038 ± 0.09 | 0.933 ± 0.077† (0.902) | 0.646 ± 0.259 (0.841) |
| $K_{mb}$ (mM) | 0.177 ± 0.024 | 0.326 ± 0.072 (0.504) | 0.175 ± 0.062 (0.348) | 0.562 ± 0.134 | 0.647 ± 0.170 (0.659) | 0.401 ± 0.264 (0.613) |
| $K_D$ (diffusion constant) | | – | 8,515 ± 4,089 (7,801) | | – | 10,353 ± 10,718 (2,270) |

Subscript a, lower-rate/higher-affinity transporter ($y^+L$); subscript b, higher-rate/lower-affinity transporter ($y^+$)

[a] Preferred model in $ECV_{304}$ cells ($F = 0.641$ [$n = 180$], $P = 0.42$); note the high SEM for $K_D$

[b] Preferred model in HUVECs ($F = 0.857$ [$n = 23$], $P = 0.37$); note the high SEM for $K_D$

*, **, *** Differences in the $V_{max}$ values of the Eadie-Hofstee plot and the Model without diffusion; † Differences in the $V_{maxb}$ values of the ECV and HUVEC Models without diffusion

equivalent results were obtained for HUVECs: $V_{maxa} = 0.103 \pm 0.012$ nM arginine/$4 \times 10^5$ cells/min, $K_{ma} = 0.017 \pm 0.002$ mM, $V_{maxb} = 0.907 \pm 0.034$ nM arginine/$4 \times 10^5$ cells/min, $K_{mb} = 0.612 \pm 0.073$ mM (i.e., the kinetic constants obtained from the model fit of Fig. 3d matched those of the fit of Fig. 3a; Table 1).

## Inhibition of Arginine Uptake by Leucine

Leucine decreased both $V_{maxa}'$ and $K_{ma}'$ of tracer uptake by the high-affinity/low-rate transporter in $ECV_{304}$ cells at all concentrations of leucine tested (Fig. 4). $V_{maxa}'$ decreased significantly until leucine concentrations reached 0.200 mM ($P < 0.0005$) and then was increased at 0.500 mM leucine (0.200 vs. 0.500 mM, $P = 0.0031$; Fig. 4a). Similarly, $K_{ma}'$ was also minimal at a concentration of approximately 0.200 mM leucine ($P < 0.005$ vs. leucine = 0 mM) and was increased at 0.500 mM leucine (0.200 vs. 0.500 mM, $P < 0.0001$; Fig. 4b). To determine the type of inhibition, the ratio $K_{ma}'/V_{maxa}'$ increased linearly with the concentration of leucine ($r^2 = 0.973$) and as both $K_{ma}'$ and $V_{maxa}'$ were decreased (i.e., neither were constant), the results suggested that the inhibition of this transporter by leucine was mixed. These data were consistent with "discoordinated inhibition" (Krupyanko 2007) with a $K_{ia} = 0.024 \pm 0.003$ and 0.159 mM when leucine was < 0.500 mM and equal to 0.500 mM, respectively.

The maximum rate of uptake ($V_{maxb}'$) of the low-affinity/high-rate transporter in $ECV_{304}$ cells was unchanged in the presence of leucine, except at 0.500 mM leucine when $V_{maxb}'$ was significantly lower ($P < 0.0001$ for all leucine concentrations versus 0.500 mM, Fig. 4a). Compared to the uninhibited reaction, $K_{mb}'$ was significantly reduced at leucine concentrations of 0.033–0.200 mM ($P < 0.05$) but was not different from the uninhibited reaction at 0.500 mM leucine ($P = 0.35$, Fig. 4b). The data suggest that such inhibition/activation was competitive for leucine concentrations of 0.033–0.200 mM and were consistent with a model of "competitive activation" (Krupyanko 2007), with $K_{ib} = 0.0.040 \pm 0.005$ and $K_{ib} = 1.554$ mM when leucine was < 0.500 and 0.500 mM, respectively. There were insufficient data to determine whether $V_{maxb}$ simply decreased linearly with increasing leucine, which would suggest uncompetitive inhibition (unassociative; Krupyanko 2007).

Nonlinear modeling suggested that leucine affected both transporters differently without either transporter being completely inhibited at any of the concentrations of leucine tested. These kinetic constants were not determined at higher leucine concentrations.

## Effect of NEM on Arginine Uptake in $ECV_{304}$ Cells

NEM (0.200 mM) incubated with $ECV_{304}$ cells for 10 min prior to the uptake of labeled arginine completely inhibited the low-affinity/higher-rate $y^+$ transporter. The result was tested using a single model of transport, which was preferred to a two-transporter model of uptake, with the results for the latter model being ambiguous and both $K_{mb}'$ and

$V_{maxb}'$ defaulting to a large value (infinity) when using GraphPad Prism (see Materials and Methods, Table 2), suggesting noncompetitive inhibition.

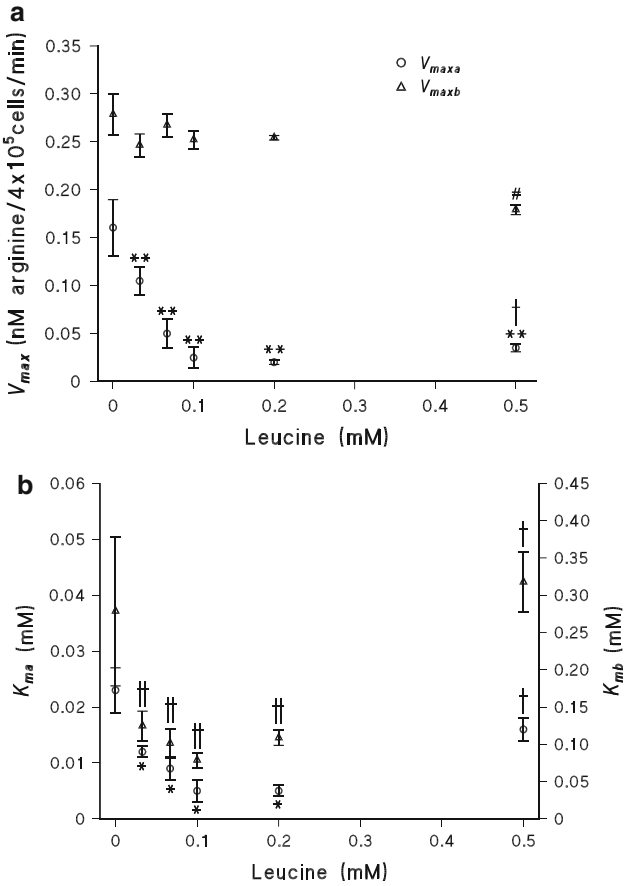


**Fig. 4** The effect of leucine on (**a**) the rate ($V_{max}'$) of 10 nM $^3$H-arginine uptake and (**b**) Michaelis constant ($K_m'$) for the individual transporters, as determined by nonlinear modeling described above. Results are mean ± SD from triplicate experiments. The experimental method is described in Materials and Methods, with the PBS test solution containing 10 nM $^3$H-arginine; various concentrations of unlabeled arginine with the concentrations of leucine as indicated in the graphs. *$P < 0.005$ and **$P < 0.0005$ versus no leucine; †$P < 0.005$ versus 0.2 mM leucine; $^{\#}P < 0.0001$ versus all other leucine concentrations; ††$P < 0.05$ versus no leucine

### Effect of BCH on Arginine Uptake in $ECV_{304}$ Cells

BCH (30 mM) was preincubated with $ECV_{304}$ cells for 60 min prior to determining tracer uptake. A two-transport model was preferred over a single-transport system ($P < 0.0001$). $V_{maxa}'$ and $K_{ma}'$ were both significantly reduced ($P < 0.0001$), whereas $V_{maxb}'$ and $K_{mb}'$ were unchanged (Table 2), suggesting that only the $y^+L$ transport was affected by BCH.

### Sodium Dependence of L-Arginine Uptake

Reducing sodium from 157 to 26 mM affected neither $V_{maxa}'$ nor $K_{ma}'$, whereas the absence of sodium decreased both constants (Table 3) (157 vs. 0 mM, $V_{maxa}$ $P < 0.001$ and $K_{ma}$ $P < 0.01$; 26 vs. 0 mM, $V_{maxa}$ $P < 0.01$ and $K_{ma}$ $P < 0.05$). $K_{ma}'/V_{maxa}'$ was unchanged, suggesting that the absence of sodium affected transport by $y^+L$ uncompetitively. $V_{maxb}'$ of the low-affinity/high-rate transporter was significantly reduced when sodium was reduced or absent ($P < 0.0005$, 26 vs. 0 mM, $P$ = ns), whereas $K_{mb}'$ was unchanged, suggesting noncompetitive inhibition of $y^+$ transport when sodium was reduced.

## Discussion

The present study used raw, rather than transformed, data in nonlinear regression analysis to characterize the kinetics of arginine uptake into cells by more than one transporter, thereby avoiding the potential difficulties and errors of methods deriving constants from the linearization of the uptake process following Michaelian kinetics (Malo and Berteloot 1991). Importantly, the approach allowed for the concentrations of both labeled and unlabeled arginine to be included and by determining changes in $V_{max}'$ or $K_m'$ was able to determine the inhibition type for either transporter in the presence of effector molecules.

**Table 2** Effect of preincubation of the inhibitors NEM (0.2 mM) and BCH (30 mM) on arginine uptake by $ECV_{304}$ cells

| | NEM | | BCH | |
|---|---|---|---|---|
| | 0 mM | 0.2 mM | 0 mM | 30 mM |
| $V_{maxa}$ (nM/4 × $10^5$ cells/min) | 0.166 ± 0.0265 (0.224) | 0.371 ± 0.011 (—) | 0.294 ± 0.029 (0.362) | 0.012 ± 0.004*** (0.010) |
| $K_{ma}$ (mM) | 0.024 ± 0.003 (0.032) | 0.059 ± 0.003 (—) | 0.026 ± 0.002 (0.030) | 0.002 ± 0.001*** (0.002) |
| $V_{maxb}$ (nM/4 × $10^5$ cells/min) | 0.268 ± 0.026 (0.3) | ~5.10 × $10^{14}$***,[a] (—) | 1.509 ± 0.071 (1.867) | 2.109 ± 0.102 (2.084) |
| $K_{mb}$ (mM) | 0.272 ± 0.083 (0.762) | ~2.08 × $10^{15}$***,[a] (—) | 0.598 ± 0.085 (1.024) | 0.602 ± 0.049 (0.595) |

Results are mean ± SEM from triplicate experiments, with robust values in parentheses

[a] After preincubation with NEM, the best fit of $V_{maxb}$ and $K_{mb}$ defaulted to a large value ($\infty$), with an ambiguous result. Hence, a single-transport model with diffusion was preferred

Difference between uptake with and without inhibitor: *** $P < 0.0001$

**Table 3** Kinetic constants for $ECV_{304}$ cells determined at various sodium concentrations

| | Sodium 157 mM | Sodium 26 mM | Sodium 0 mM |
|---|---|---|---|
| $V_{maxa}$ (nM arginine/4 × $10^5$ cells/min) | 0.142 ± 0.027 (0.184) | 0.201 ± 0.036 (0.236) | 0.100 ± 0.007**† (0.118) |
| $K_{ma}$ (mM) | 0.016 ± 0.002 (0.019) | 0.018 ± 0.003 (0.021) | 0.011 ± 0.001*†† (0.012) |
| $K_{ma}/V_{maxa}$ | 1.09 ± 0.05 × $10^{-3}$ | 0.92 ± 0.04 × $10^{-3}$ | 1.07 ± 0.02 × $10^{-3}$ |
| | [0.99 to 1.20 × $10^{-3}$] | [0.82 to 1.00 × $10^{-3}$] | [1.03 to 1.11 × $10^{-3}$] |
| $V_{maxb}$ (nM arginine/4 × $10^5$ cells/min) | 0.660 ± 0.022 (0.695) | 0.313 ± 0.028*** (0.356) | 0.350 ± 0.009*** (0.403) |
| $K_{mb}$ (mM) | 0.193 ± 0.027 (0.283) | 0.210 ± 0.073 (0.400) | 0.204 ± 0.020 (0.315) |
| $K_{mb}/V_{maxb}$ | 2.93 ± 0.46 × $10^{-3}$ | 6.70 ± 2.72 × $10^{-3}$ | 5.85 ± 0.54 × $10^{-3}$ |
| | [2.01 to 3.84 × $10^{-3}$] | [1.31 to 12.08 × $10^{-3}$] | [4.78 to 6.91 × $10^{-3}$] |

Results are mean ± SEM from triplicate experiments, with robust value in parentheses and 95% CI for the slope in brackets

Significant differences: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ for 0 or 26 mM versus 157 mM sodium; † $P < 0.005$, †† $P < 0.0005$ for 0 versus 26 mM sodium

The results show that (1) the effect of the unlabeled amino acid on the rate of uptake of the "trace" labeled amino acid was not linear and depended on the relative concentrations of unlabeled to labeled amino acid; (2) the theoretical plot of the total rate of uptake, derived from the kinetic constants of arginine uptake estimated from Eadie-Hofstee plots, significantly overestimated the experimental data; and (3) when the constants were estimated from the nonlinear modeling (Malo and Berteloot 1991), there was good agreement. The results suggest that values of kinetic constants calculated from linearized data reported for cationic amino acids in the literature (Devés and Boyd 1998; Mann et al. 2003) may have overestimated the true values. Although Malo and Berteloot (1991) developed this approach for glucose uptake by more than one transporter, as far as we are aware this is the first report to show the discrepancy between theoretical and experimental data for amino acid uptake by more than one transporter.

Nonlinear regression analysis allowed comparison between models of one or two transporters, the presence of diffusion (Malo and Berteloot 1991) and the possibility of uptake by a single transporter with multiple binding sites (Chenu and Bertloot 1993). The results consistently determined that, in the absence of inhibitors, a model of uptake by two independent transporters was preferred over other models. This conclusion was supported by curvilinear Eadie-Hofstee plots using transformed data.

Studies have suggested that the uptake in HUVECs was facilitated by a single high-affinity transporter with a diffusion component, rather than two simultaneously operating transporters (Arancibia-Garavilla et al. 2003; Speake et al. 2003); however, expression data suggest that additional cationic transporters were operational in these cells (Rotmann et al. 2007), and thus the activity ascribed to the $y^+L$ transporter could in fact be ascribed to more than one such transporter (Arancibia-Garavilla et al. 2003).

Differences between the results of various studies may be partly explained by variations in study methodology as studies have determined uptake in the presence of labeled amino acid alone (Sobrevia et al. 1995; Rotoli et al. 2005; Brunini et al. 2006), specified the activity but not the concentration of trace labeled amino acid (Closs et al. 1993a; Casanello and Sobrevia 2002; Hardy and May 2002; Arancibia-Garavilla et al. 2003; Signorello et al. 2003; Martín et al. 2006; Rotmann et al. 2007) and reported results from studies using 0.1–0.2 μM (Ayuk et al. 2002; Schlaich et al. 2004) to 50 μM (Durante et al. 1996) labeled arginine in the presence of fixed or varying concentrations of unlabeled substrate or inhibitors. Our data showing nonlinear uptake of tracer in the presence of unlabeled amino acid underscore the importance of including the concentrations of both the labeled and unlabeled amino acids when determining the rate of uptake.

Uptake by diffusion has been demonstrated as the nonsaturable uptake of labeled arginine (0.05 mM), measured in the presence of excess unlabeled arginine (10 mM) (Speake et al. 2003; Martín et al. 2006). Subtracting this nonsaturable uptake from the total transport (Dall'Asta et al. 2000; Arancibia-Garavilla et al. 2003) may exceed the contribution of the higher-affinity/lower-rate transporter ($y^+L$) (Dall'Asta et al. 2000), which may be among the reasons that this transporter has been overlooked (Devés et al. 1992). Subtracting the diffusion component from transformed data results in the loss of statistical information, whereas when using the nonlinear approach, such information is retained, allowing error in the calculated kinetic constants to be determined (Malo and Berteloot 1991). In our experiments using 10 nM labeled arginine, the inclusion of the diffusion term did not improve the fit of the models and the contribution of diffusion to the total counts was insignificant. This does not suggest that diffusion or the contribution of a nonsaturable

component is not important but, rather, that under the experimental conditions used, this contribution was small.

## Inhibitors and Effector Molecules

The nonlinear approach makes no assumptions regarding the type of inhibition or activation of transport by effector molecules. We investigated the inhibition of arginine uptake by leucine, which has been used to differentiate between $y^+L$ and $y^+$ transport; NEM, which inhibits $y^+$ transport; and the neutral amino acid analogue BCH, an inhibitor of sodium-independent transport (White et al. 1982). Furthermore, the effects of sodium on uptake were determined.

### *Leucine*

Studies have used fitted models of competitive inhibition of one or both transporters to experimental data when determining the effects of neutral amino acid on cationic amino acid uptake (Devés et al. 1992; Devés and Boyd 1998; Hardy and May 2002; Rotoli et al. 2005). As the side chain of leucine and other neutral amino acids differs from that of arginine and lysine, it appears that models which included noncompetitive and uncompetitive inhibition were not tested. Indeed, data showing $K_i$ coinciding with $I_{50}$ (Angelo et al. 2005) would be consistent with noncompetitive or uncompetitive inhibition of the transport of leucine (Cheng and Prusoff 1973).

Our data showing that leucine affected $y^+L$ transport by mixed inhibition ("discoordinated inhibition") and $y^+$ transport by "competitive activation" (Krupyanko 2007) are in contrast to previous studies which determined that leucine competitively inhibited $y^+L$ transport in erythrocytes (Devés et al. 1992), HUVECs (Arancibia-Garavilla et al. 2003) and platelets (Signorello et al. 2003). However, these reports did not determine inhibition by leucine at low and physiological concentrations.

Although leucine did not completely inhibit transport at any of the concentrations tested, other studies, using higher leucine concentrations (1–10 mM leucine in the presence of sodium), determined uptake of leucine-sensitive transport (i.e., the contribution of $y^+L$ transport) by subtracting leucine-insensitive uptake (i.e., the contribution by $y^+$) from the total uptake (Mendes-Ribeiro et al. 1999; Arancibia-Garavilla et al. 2003; Martín et al. 2006; Rotmann et al. 2007). However, as stressed above, the results of such studies would depend on the relative concentrations of the added labeled tracer versus the unlabeled arginine present and were included in the models used in our study.

Different neutral amino acids affect arginine uptake differently (Closs et al. 1993a; Bröer et al. 2000; Rotmann et al. 2007), and results may not be consistent if, for example, leucine was replaced by glutamine (Ayuk et al. 2002; Speake et al. 2003). In addition, there appear to be no data suggesting that leucine and glutamine inhibit cationic transport in the same manner, and such studies could be tested using this nonlinear modeling approach.

### *NEM and BCH*

The nonlinear modeling approach determined that the $y^+L$ transporter was unaffected by NEM, whereas the $y^+$ transporter was completely inhibited, which was consistent with the literature (Devés and Boyd 1998; Babu et al. 2003). Almost three decades ago, BCH was developed as a model substrate for the sodium-independent system for neutral amino acids (White and Christensen 1982). Interestingly, the results of the present study show that BCH significantly reduced the activity of the $y^+L$ transporter without affecting the $y^+$ transporter. In the absence of a $y^+L$ transporter inhibitor, BCH or structurally related analogues may be of interest in the quest to obtain a selective inhibitor for this transporter.

### *Sodium Dependence of Arginine Transport*

Cationic $y^+L$ and $y^+$ amino acid transport is accepted as being independent of sodium concentration (Devés and Boyd 1998; Mann et al. 2003; Bröer 2008). White et al. (1982) originally noted inhibition of cationic amino acid transport by neutral amino acids, such as leucine, glutamine, methionine and, to a lesser extent, homoserine and serine (see also Devés et al. 1992). Such sodium-dependent inhibition of cationic transporters was used to identify the $y^+L$ transporter (Devés et al. 1992) and has been used to differentiate this transporter from the $y^+$ transporter (Mendes-Ribeiro et al. 1999; Rojas and Devés 1999; Arancibia-Garavilla et al. 2003; Martín et al. 2006; Rotmann et al. 2007). However, data suggest that neutral amino acids also inhibit uptake of cationic amino acids by $y^+$ transport (Christensen 1990), with some 30% of lysine uptake into HUVECs (Hardy and May 2002) and bovine aortic endothelial cells (Durante et al. 1996) being sodium-dependent. The latter could be explained if other transporters were operational in these cells, but such discrepancies in the literature highlight the difficulties in comparing cationic transport studies and appear to be similar to those noted for epithelial cells (reviewed by Bröer 2008).

Although it was suggested that sodium occupies the site normally occupied by the $\omega$-amino group of cationic amino acids to facilitate neutral amino acid transport (Christensen and Antonioli 1969), if sodium was already occupying the $\omega$-amino group site, it is not clear how these transporters facilitate cationic amino acid uptake. Our results may

suggest that sodium ions are necessary for the conformational integrity of the transporters or were required for transport of arginine; i.e., sodium interacts with the arginine–transporter complex.

Three concentrations of sodium were used to determine its effects on arginine uptake in $ECV_{304}$ cells. In contrast to earlier findings, the results suggest that $y^+L$ transport was relatively unaffected by sodium concentration unless sodium was absent when transport was uncompetitively inhibited. In contrast, the rate of arginine uptake by the $y^+$ transporter was significantly reduced as sodium was decreased, without affecting the affinity of this transporter (i.e., noncompetitively inhibited). The results suggest that the absence of sodium would decrease the total rate of uptake by both transporters but would only affect the affinity of the $y^+L$ transporter. These data underscore the advantage of measuring uptake over a wide range, not single concentrations of unlabeled substrate, and to determine the kinetic constants of both transporters, which is possible using this nonlinear approach.

### Study Limitations

Although the origin of $ECV_{304}$ cells is controversial (Brown et al. 2000; Drexler et al. 2002; Mann et al. 2003), these cells express hCAT1 ($y^+$) and $y^+$LAT2 ($y^+L$) transporters (Rotmann et al. 2007) and were therefore used as a model to determine uptake by more than one transporter.

We depleted cells of arginine for 24 h (Sessa et al., 1990), with other studies using times between 1 and 24 h (Mann et al. 2003). Intracellular arginine levels appear to be maintained in HUVECs over this time (Mann et al. 2003), and we did not determine whether shorter depletion periods affected the rate of arginine uptake. The initial rate of arginine uptake was then measured for 30 s, assuming that both the conversion of labeled arginine to metabolites, which may be exported, and the export of arginine via trans-stimulation (Flores et al. 2003) were insignificant. Furthermore, our results were in agreement with the literature (Mann et al. 2003).

Although HUVECs also express other transporters, hCAT1, -2B, y + LAT1 and -2 (Rotmann et al. 2007), under the experimental conditions, the study showed statistically that a two-transporter model was preferred over those of a single- or three- (six unknowns) transporter model, with or without multiple binding sites. The possibility of a single transporter modified by the binding of a regulatory subunit or by forming a dimeric transporter (Oulianova and Berteloot 1996) may explain many of the findings and will be the subject of future investigations.

The effects of sodium on uptake of arginine in the presence of leucine, particularly at 1–10 mM leucine concentrations, and other neutral amino acids remain to be determined by this method. In the absence of data measuring labeled arginine uptake in the presence of millimolar leucine concentrations, at this time we have no explanation for the observation of a change in the rate of arginine uptake at 0.5 mM leucine. As $V_{maxb}$ appeared to decrease with increasing leucine concentrations, the inhibition may simply be uncompetitive (unassociative; Krupyanko 2007); but there were insufficient data to substantiate this possibility. Moreover, the possibility that leucine and sodium affect the membrane potential (or pH in the case of sodium), thereby influencing arginine transport, was not investigated in our study and cannot be excluded (Devés and Boyd 1998).

## Summary

This nonlinear modeling approach to characterize the uptake of arginine in cells using raw, rather than transformed data, avoids the difficulties and errors of methods deriving constants from the linearization of uptake processes following Michaelian kinetics. Moreover, the model allows for more than one transporter to be modeled, accounts for the concentrations of both labeled and unlabeled arginine and allows the constants to be determined over a range of unlabeled, not single, substrate or inhibitor concentrations, without making assumptions regarding the type of inhibition/activation present. The theoretical models of total uptake closely matched the experimental data, uptake curves could be obtained for the individual transporters and the effects of inhibitors and sodium on the individual transporters determined. In conclusion, we have shown that the use of a nonlinear regression modeling approach may more accurately represent the kinetics of cationic amino acid uptake, activation and inhibition into cells and, hence, provides explanations for discrepancies in the literature.

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