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TRANSPORT OF NITRIC OXIDE SYNTHASE INHIBITORS THROUGH CATIONIC AMINO ACID CARRIERS IN HUMAN ERYTHROCYTES

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Abstract—The interaction of arginine analogues, which are known to inhibit nitric oxide synthase, with two cationic amino acid transporters of human erythrocytes (systems y^+ and y^+L) was studied. Arginine and relevant analogues [N^G-monomethyl-L-arginine (L-NMMA); N^G-monomethyl-D-arginine (D-NMMA) and N^G-nitro-L-arginine (L-NOARG)] were found to inhibit labeled lysine influx into intact erythrocytes. As expected, the pattern of inhibition reflected the contribution of the two distinct transport systems. All analogues showed a higher affinity for system y^+L than for system y^+. The half-saturation (inhibition) constants estimated for systems y^+ and y^+L (±SEM) were (μM): L-arginine, 55.7 ± 5.4 and 2.4 ± 0.1; L-NMMA, 151 ± 13 and 7.5 ± 0.5; D-NMMA, 2660 ± 404 and 269 ± 25; L-NOARG, 9414 ± 169 and 594 ± 35. The transport properties of the analogues were investigated using an assay based on the *trans*-stimulation of lysine efflux. The addition of saturating concentrations of unlabeled analogues to the external medium stimulated efflux of labeled lysine through systems y^+L and y^+, showing that the analogues can enter the cell through these pathways.

Key words: transport; nitric oxide; inhibitors; amino acids; arginine; erythrocytes

Bogle *et al.* [1] have shown that of the arginine analogues known to be inhibitors of nitric oxide synthesis [2, 3], some also inhibit the transport of this amino acid across the plasma membrane. Thus, in cultured vascular endothelial cells, the influx of labeled arginine (50 μM) was readily inhibited by L-NMMA§ and L-NIO, but not over the concentration range studied by L-NOARG or L-NAME. This raised the obvious question of how these pharmacologically important molecules reach their intracellular site of action.

In a recent publication, Schmidt *et al.* [4] addressed this question measuring the transport of radiolabeled L-NMMA and L-NOARG into cultured porcine aortic endothelial cells. It was concluded that L-NMMA was transported by two systems differing in affinities (K_m values, estimated from uptake rates measured over a period of 30 min were 4 and 368 μM). The total flux of L-NMMA was inhibited by 1 mM unlabeled L-lysine and L-ornithine, but not by L-leucine and L-isoleucine (although L-phenylalanine and L-glutamine inhibited by approximately 40% at this concentration). The flux of L-NOARG was consistent with transport through a single system that was strongly inhibited by L-leucine and L-isoleucine, but not L-arginine and L-ornithine. Thus, L-NMMA and L-NOARG were found to interact with at least three different transport systems in endothelial cells.

Separate routes for the transport of nitric oxide synthesis inhibitors have also been proposed in the case of macrophages [5, 6]. Activation of macrophage J774 cells with lipopolysaccharide (LPS) resulted in an increase in the maximum velocity of L-NMMA transport, whereas L-NOARG transport remained unchanged. Moreover, the specificities of these two transport activities in unstimulated cells were found to differ. L-arginine inhibited L-NMMA transport (K_i = 0.39 mM), but was a poor inhibitor of L-NOARG transport (approximately 10% inhibition at 5 mM arginine). L-NAME showed the opposite preference [5]. Similar results were obtained with macrophage RAW 264.7 cells. Activation of these cells by addition of lipopolysaccharide and interferon-γ increased L-NMMA uptake without affecting the transport of L-NOARG. L-NMMA transport was inhibited by L-arginine and L-lysine, but only weakly by L-isoleucine. Conversely, L-NOARG transport was strongly inhibited by unlabeled L-isoleucine, but weakly by L-arginine and L-lysine [6].

Parallel pathways for cationic amino acids, showing different substrate specificities, affinities, and cation dependence, have been described in a variety of cell membranes [7–10], and more recently, two transporters (y^+ and y^+L) have been kinetically characterized in red cells and in placental trophoblasts [11–13]. Activated human T lymphocytes, which produce nitric oxide also appear to have these two transporters [14]. System y^+ exhibits a relatively low affinity for lysine (K_m = 100–200 μM at physiological membrane potential), and its specificity is restricted to cationic amino acids; system y^+L recognizes lysine with higher affinity (K_m = 10–30 μM) and can also operate in the presence of Na^+ as a carrier for neutral amino acids. Both in human erythrocytes and placental membranes, system y^+ exhibits an approximately 10-fold higher capacity than system y^+L, and thus it accounts for a large fraction of the flux when transport is measured at high concentrations. At very low substrate concentrations, both systems contribute about equally to substrate influx.

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§ Abbreviations: L-NMMA, N^G-monomethyl-L-arginine; D-NMMA, N^G-monomethyl-D-arginine; L-NIO, N^G-ethylimino-L-ornithine; L-NOARG, N^G-nitro-L-arginine; L-NAME, N^G-nitro-L-arginine methyl ester; and NEM, *N*-ethylmaleimide.

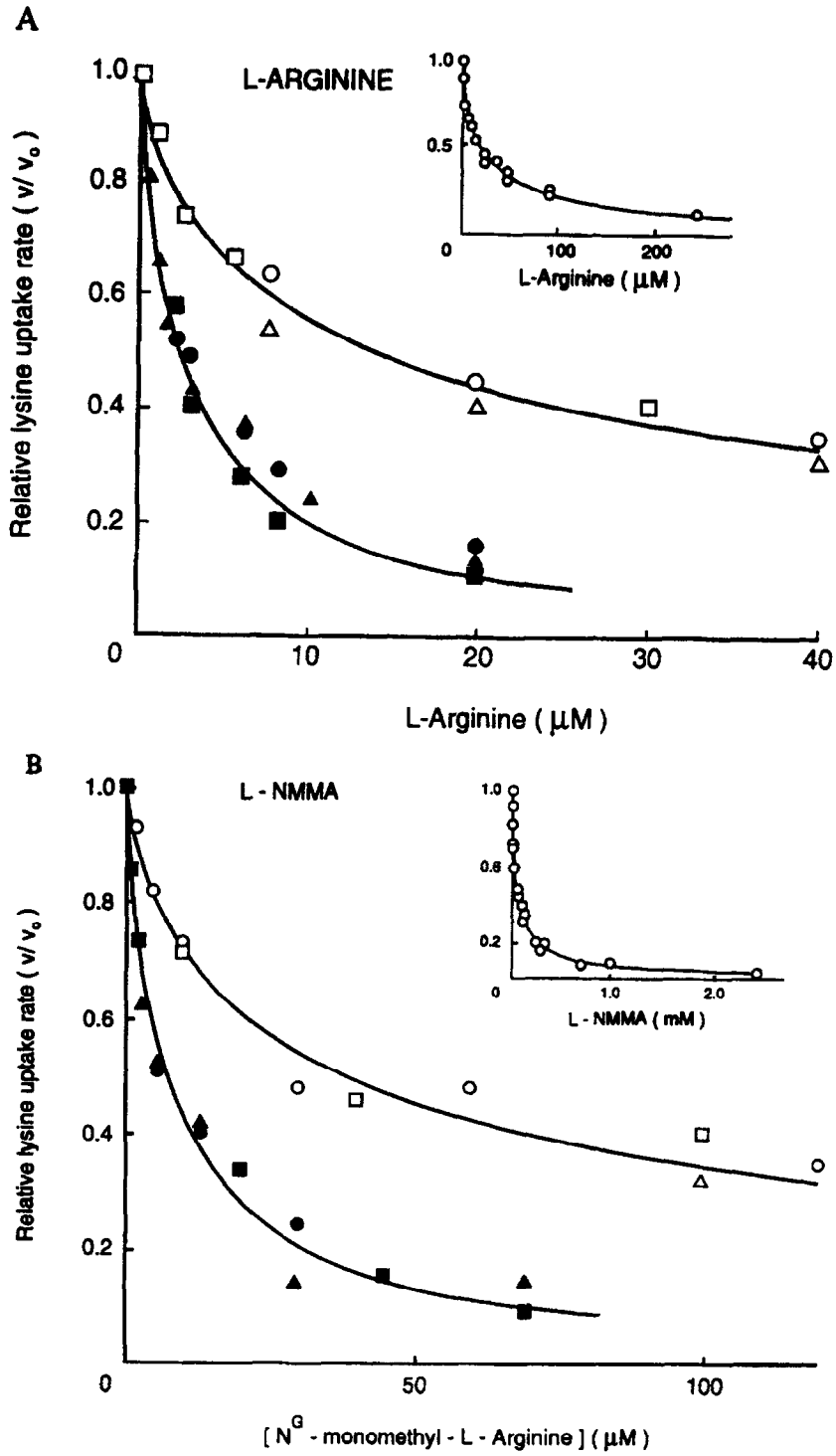

Fig. 1. Relative rates of L-[^{14}C]lysine entry in the presence of various concentrations of unlabeled L-arginine (A), L-NMMA (B) and L-NOARG (C) in the external medium in intact (open symbols) and NEM-treated (closed symbols) cells. Labeled lysine concentration was kept fixed at 1 µM. All rates were calculated from six data points, and the different symbols represent different individual cell samples. Entry rates (±SEM) in the absence of unlabeled analogue (v_o) ranged from 0.061 ± 0.004 to 0.11 ± 0.003 µmol/L cells/min (intact cells) and from 0.035 ± 0.0008 to 0.057 ± 0.0007 µmol/L cells/min (NEM-treated cells). Curves have been fitted to the data by non-linear regression analysis according to equation (1) (intact cells) or equation 2 (NEM-treated cells). Insets show the results obtained for a wider concentration range (intact cells).

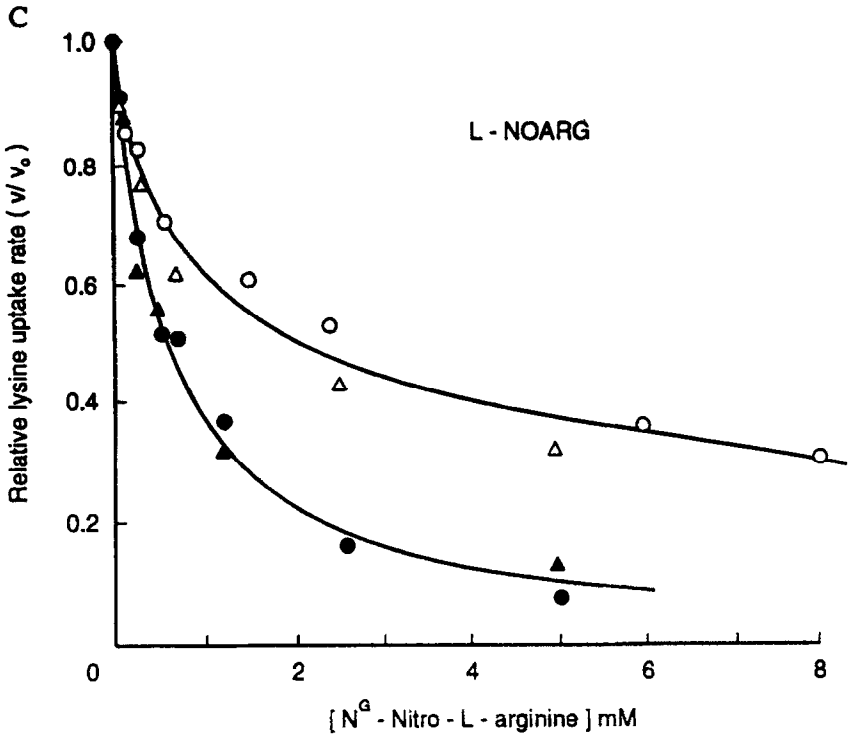

Fig. 1. Continued.

Here we explore the interaction of systems y^+ and y^+L with L-arginine and arginine analogues that inhibit nitric oxide synthase. The activities of systems y^+ and y^+L can be conveniently dissected in erythrocytes by the use of the sulfhydryl reagent NEM [12], which inhibits system y^+ selectively. Moreover, the transport properties of unlabeled analogues can be inferred from their effects on the unidirectional flux of a single labeled substrate (L-lysine). Recently, this method was used successfully to characterize the substrate specificity of system y^+L in these cells [15].

MATERIALS AND METHODS

Experimental

Uniformly labeled L-[^{14}C]lysine was purchased from Amersham (approximately 12 GBq/mmol); unlabeled amino acids, NEM and L-NOARG were from Sigma; dibutylphthalate was from Merck; L-NMMA and D-NMMA were from Calbiochem.

Details of the methods have been described previously [11, 12, 15]. Briefly, human peripheral blood was obtained from donors and used immediately following overnight incubation with 150 mM NaCl, 4 mM KCl, 5 mM sodium phosphate, pH 6.8, to reduce the intracellular amino acid pool. Selective inactivation of systems y^+ was achieved using NEM. Cells (2.5% hematocrit) were preincubated for 10 min at 25° with 0.2 mM NEM, and the reaction was stopped by the addition of excess 2-mercaptoethanol followed by washing of the cells with buffered saline solution.

The entry of L-[^{14}C]lysine was followed at 37°, 10% hematocrit, by determining the time course of labeled lysine uptake. All determinations were performed in duplicate and rates were estimated from linear regression analysis of six time points (up to 4 min); inhibitors were added simultaneously with the labeled substrate.

For the measurement of exit, cells were preloaded initially by incubation with labeled lysine (6 μM) at 37° and 50% hematocrit. Aliquots of loaded cells (0.3 mL) were added at 10% hematocrit to assay buffer with or without unlabeled analogues. Samples (0.9 mL) were withdrawn at intervals and rapidly centrifuged in tubes containing dibutylphthalate. The radioactivity in the supernatant was determined by scintillation counting. All determinations were performed in duplicate runs, and rates (±SEM) were calculated by linear regression from six time points [15].

Kinetic analysis

The basis of the analysis used has been described in previous publications [11, 12]. The following equations describing the relative rate of entry of substrate S, in the presence (v) and in the absence (v_o) of analogue I, were used to calculate the inhibition constants for the analogues. The rate equations have been simplified by assuming that the substrate concentration is very low compared to its half-saturation constant ([S] << K_m); all experiments were designed to match this requirement.

Relative rate of entry in the presence or absence of an unlabeled analogue for two parallel transport systems (y^+ and y^+L).

$$v/v_o = \frac{F/(1 + [I]/K_{iYL}) + 1/(1 + [I]/K_{iY})}{F + 1} \quad (1)$$

K_{iY} and K_{iYL} represent the inhibition constants for the analogue (I) for systems y^+ and y^+L, respectively. Constant F is the ratio of the substrate (lysine) transport

parameters for the two systems $[(V_{maxYL})\ (K_{mY})/(K_{mYL}/V_{maxY})]$ and was shown previously to equal one [11].

Relative rate of entry in the presence or absence of an unlabeled analogue for one transport system (y^+L).

$$v/v_o = 1 + [I]/K_{iYL} \quad (2)$$

RESULTS AND DISCUSSION

Arginine was found to inhibit radioactive lysine influx into intact erythrocytes and, as predicted, this inhibition showed biphasic behavior reflecting the combined contribution of the high (system y^+L) and low (system y^+) affinity transport system (Fig. 1A). Following reaction of the cells with NEM, which inactivates system y^+ selectively [12], the pattern of inhibition by unlabeled L-arginine was simplified and was well described by a single high-affinity system (Fig. 1A). This analysis was extended to structural analogues of arginine, which have been used to investigate the biological role of nitric oxide. L-NMMA (Fig. 1B) behaved similarly to arginine, whereas L-NOARG (Fig. 1C) was a weaker inhibitor; nevertheless, this analogue was able to inhibit lysine influx through systems y^+L in the concentration range studied. Table 1 shows the values of the kinetic constants that have been calculated to fit equation 1 or equation 2 to the data for the analogues studied, including the D-stereoisomer of NMMA. The two independent estimates of K_{iYL} for the arginine analogues were in good agreement with each other, in strong support of the model underlying the analysis. The table provides evidence that, for all analogues, system y^+L had a higher apparent affinity (10- to 20-fold greater) than did system y^+; for arginine, the apparent affinity was 23-fold greater.

In further experiments, we used an assay, based on the *trans*-stimulation of radioactive lysine efflux by unlabeled analogues, to determine whether the inhibitory interactions described above reflected translocation or simply binding of the analogues to the transporters [16]. Previous work has shown that for system y^+L return of the free carrier is rate-limiting for unidirectional lysine efflux [15]. Thus, the effect of analogues, present in the external medium, upon the flux of labeled substrate provides information regarding the transport properties of the carrier-analogue complex. As Fig. 2 shows, the addition of unlabeled analogues to the external medium stimulated efflux of labeled lysine through system y^+L markedly. Unlabeled L-lysine, which had been found previously to stimulate unidirectional efflux by approximately 6-fold [15], was used here as a reference. Since, as predicted by the carrier model [16], *trans*-stimulation is concentration dependent and follows Michaelis–Menten kinetics saturating at high concentrations [15], the concentrations of all analogues used here were chosen to satisfy this condition. Figure 3 summarizes the results and includes data on both D- and L-stereoisomers of lysine, arginine and NMMA (K_{iy^+L} for D-lysine [10] and D-arginine is 150 and 181 ± 91 μM, respectively). The dotted line on the histograms refers to the average relative rate of efflux in the absence of unlabeled analogue; the rate in the presence of the analogues was normalized to the value in the presence of 0.5 mM L-lysine. L-Lysine stimulated the flux by 6.0 ± 0.8 (N = 6). As may be readily seen, in each instance, and with all the nitric oxide inhibitors tested, there was clear evidence of *trans*-stimulation. Since the relative maximum acceleration of efflux caused by the two analogues measures their relative maximum rates of entry [15, 16], this indicates that all the analogues are translocated through system y^+L.

As shown in Fig. 4, *trans*-stimulation was also observed for system y^+ (NEM-sensitive rate), but the degree of acceleration was considerably smaller. L-Lysine, L-NMMA and L-arginine stimulated the rate by approximately 1.4- to 1.5-fold whereas with L-NOARG the ratio was only 1.2. However, since a non-transported inhibitor would be expected to fully inhibit the rate of exit, it can be concluded that all of the analogues tested can be translocated by system y^+. As reported in an earlier publication [15], the rate of exit of labeled L-lysine via system y^+L (under zero-*trans* conditions) was lower than the corresponding activity for system y^+. The apparently low activity of system y^+L in efflux experiments suggests competition between L-[^{14}C]lysine and the residual intracellular amino acid pool; endogenous amino acids are not expected to have the same effect on system y^+, which has a lower affinity and a more restricted substrate specificity.

In conclusion, our studies demonstrated that both systems (y^+ and y^+L) are able to bind and translocate L-arginine and the pharmacologically relevant amino acid analogues. In agreement with previous observations for L-lysine [11, 12], all analogues were found to interact more strongly with system y^+L, which has been defined

Table 1. K_i for *cis*-inhibition of L-lysine influx through systems y^+ and y^+L

	K_i (μM)		
	Intact cells		NEM-treated cells
Analogue	y^+	y^+L	y^+L
L-Arginine	55.7 ± 5.4	3.2 ± 0.4	2.4 ± 0.1
L-NMMA	151 ± 13	9.9 ± 1.3	7.5 ± 0.5
D-NMMA	2660 ± 404	196 ± 31	269 ± 25
L-NOARG	9414 ± 169	484 ± 71	594 ± 35

Inhibition constants (K_i ± SEM) were calculated by non-linear regression analysis of the data according to equation 1 (intact cells) or equation 2 (NEM-treated cells) as explained in Materials and Methods and the legend of Fig. 1. Intact cells: L-arginine (N = 16), L-NMMA (N = 18), D-NMMA (N = 13), and L-NOARG (N = 12). NEM-treated cells: L-arginine (N = 18), L-NMMA (N = 15), D-NMMA (N = 10), and L-NOARG (N = 12).

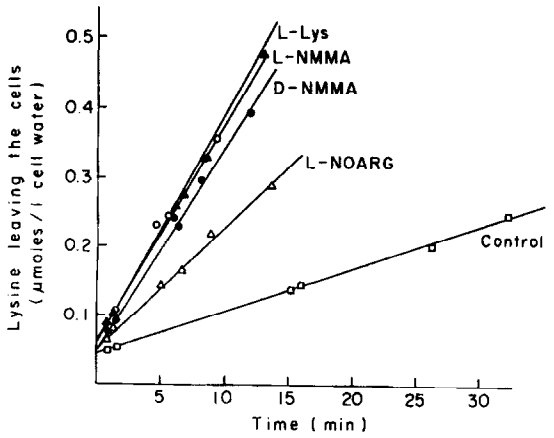

Fig. 2. L-[^{14}C]Lysine exit from NEM-treated erythrocytes in the absence (□) or presence of unlabeled 1 mM L-NMMA (▲), 2 mM D-NMMA (●), 5 mM L-NOARG (△) and 0.5 mM L-lysine (○). The internal concentration of L-[^{14}C]lysine at the beginning of the experiment was 4.4 μM, and the exit rate (±SEM) in the control was 6.2 ± 0.1 nmol/L cell water/min.

as a high-affinity and low-capacity system. The observation that system y^+ recognizes L-NOARG with very low affinity (K_i = 9414 ± 169 μM) is consistent with previous reports in endothelial cells [1, 4, 17] and macrophages [5, 6], and therefore the finding that this drug interacts with system y^+L is particularly interesting. As mentioned at the beginning of the article, in studies with endothelial cells [4] and macrophages [6], L-NOARG has been shown to enter the cells via a neutral amino

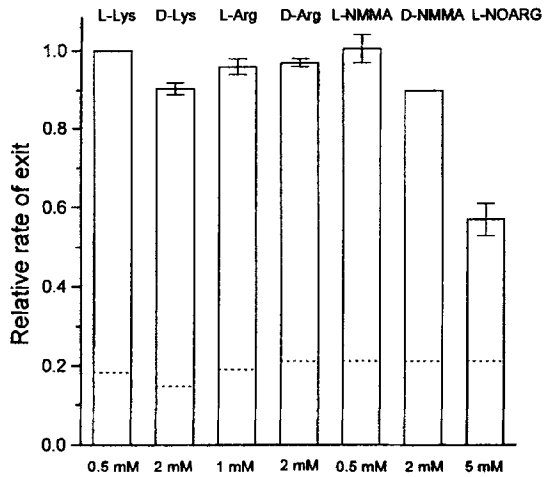

Fig. 3. Effects of arginine analogues present in the external medium on the rate of exit of L-[^{14}C]lysine in cells treated with NEM (y^+L system). Rates were normalized using as reference the rate measured in the presence of 0.5 mM L-lysine. Mean values for two rate determinations are given; error bars indicate the range of these determinations. Rates were calculated from six time points; thus each average rate is calculated on the basis of twelve individual determinations. The dotted line marks the average value for the relative rate of efflux measured in the absence of amino acid. The ratio of the rates in the presence or absence of L-lysine (±SD) was 6.0 ± 0.8 (N = 6). The internal concentration at the beginning of the experiment ranged from 3.8 to 4.6 μM and the control rate from 5 ± 0.176 to 13.5 ± 0.55 nmol/L cell water/min.

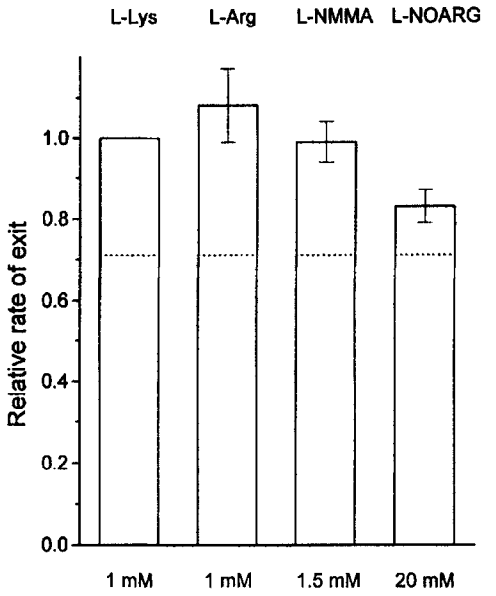

Fig. 4. Effects of arginine analogues present in the external medium on the NEM-sensitive L-[^{14}C]lysine efflux (y^+ system). Rates were normalized using as reference the rate measured in the presence of 1.0 mM L-lysine. Mean values for two rate determinations are given; error bars indicate the range of these determinations. NEM-sensitive rates were obtained by subtracting the rates measured in the presence of NEM from the total efflux rates. Each rate was calculated from six time points; thus, each average rate is calculated on the basis of twenty-four individual determinations. The dotted line marks the average value for the relative rate measured in the absence of amino acid. The ratio of the rates in the presence or absence of L-lysine was 1.45 and 1.33 for each cell sample, and the corresponding control rates (±SEM) were 143 ± 3.4 and 92 ± 4.9 nmol/L cell water/min. The internal concentration at the beginning of the experiment was 4 μm.

acid carrier that is inhibited by leucine and isoleucine, but not by lysine or arginine. This carrier is distinct from system y^+L, which recognizes L-leucine and L-lysine with comparable affinities. The apparent affinity of L-NOARG for the neutral amino acid transporter in endothelial cells (K_i = 609 μM) [4] is similar to that measured for system y^+L in our studies. In macrophages the apparent inhibition constant was 200–300 μM [5, 6].

Resolving the actual contributions of systems y^+ and y^+L to the overall rates of transport of these drugs under physiological conditions would require measuring the transport of labeled drugs in the presence of plasma concentrations of competing amino acids. It is difficult to extrapolate from *in vitro* (zero-*trans*) kinetics to make *in vivo* predictions, since in the latter case both systems will be exposed to multiple substrates at different concentrations on both sides of the membrane. However, the fact that system y^+ has an approximately 10-fold higher maximum velocity and a more restricted substrate specificity than system y^+L [12, 15] would suggest that, in red cells, it should make a more important contribution to flux at physiological concentrations. The relative importance of these routes in different tissues clearly will depend upon the transporters expressed within a given cell type.

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