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Dibasic amino acid interactions with Na^+-independent transport system asc in horse erythrocytes. Kinetic evidence of functional and structural homology with Na^+-dependent system ASC

Daron A. Fincham [a], D. Keith Mason [b] and James D. Young [a]

[a] *Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories* and [b] *Equine Hospital, The Royal Hong Kong Jockey Club, Shatin Racecourse, Shatin, New Territories (Hong Kong)*

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Amino acid transport in horse erythrocytes is regulated by three co-dominant allelomorphic genes coding for high-affinity transport activity (system asc_1), low-affinity transport activity (system asc_2) and transport-deficiency, respectively. The asc systems are selective for neutral amino acids of intermediate size, but unlike conventional system ASC, do not require Na^+ for activity. In the present series of experiments we have used a combined kinetic and genetic approach to establish that dibasic amino acids are also asc substrates, systems asc_1 and asc_2 representing the only mediated routes of cationic amino acid transport in horse erythrocytes. Both transporters were found to exhibit a strong preference for dibasic amino acids compared with neutral amino acids of similar size. Apparent K_m values (mM) for influx via system asc_1 were L-lysine (9), L-ornithine (27), L-arginine (27), L-alanine (0.35). Corresponding V_{max} estimates (mmol/l cells per h, 37 °C) were L-lysine (1.65), L-ornithine (2.15), L-arginine (0.54), L-alanine (1.69). Apparent K_m values for L-lysine and L-ornithine influx via system asc_2 were ≈ 90 and > 100 mM, respectively, with V_{max} values > 2 and > 1 mmol/l cells per h, respectively. Apparent K_m and V_{max} values for L-alanine uptake by system asc_2 were 14 mM and 6.90 mmol/l cells per h. In contrast, L-arginine was transported by system asc_2 with the same apparent K_m as L-alanine (14 mM), but with a 77-fold lower V_{max}. This dibasic amino acid was shown to cause *cis*- and *trans*-inhibition of system asc_2 in a manner analogous to its interaction with system ASC, where the side-chain guanidinium group is considered to occupy the Na^+-binding site on the transporter. Concentrations of extracellular L-arginine causing 50% inhibition of zero-*trans* L-alanine influx and half-maximum inhibition of L-alanine zero-*trans* efflux were 14 mM (extracellular L-alanine concentration 15 mM) and 3 mM (intracellular L-alanine concentration 15.5 mM), respectively. We interpret these observations as evidence of structural homology between the horse erythrocyte asc transporters and system ASC. Physiologically, intracellular L-arginine may function as an endogenous inhibitor of system asc_2 activity.

Introduction

Membrane transport of amino acids in erythrocytes is characterised by extensive interspecies and intraspecies diversity. This heterogeneity provides unique experimental material for the

Abbreviations: Mops, morpholinepropanesulphonate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate.

Correspondence: J.D. Young, Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.

study of functional and evolutionary relationships between different amino acid transport mechanisms. In human erythrocytes, transport of L-cysteine for intracellular glutathione biosynthesis is mediated largely by system ASC [1,2], an amino acid transporter with a widespread species and tissue distribution [3,4]. This system, which is also present in avian erythrocytes [5] and rabbit reticulocytes [6], has a high-affinity for neutral amino acids of intermediate size and requires Na^+ for activity. In contrast, L-cysteine transport in horse erythrocytes is mediated by high-affinity and low-affinity allelic variants of a newly discovered amino acid transporter designated system asc [7,8]. System asc has a similar substrate specificity to system ASC, but is Na^+-independent. At physiological temperature the high-affinity transporter (designated system asc_1) operates preferentially in an exchange mode. In contrast, the low-affinity carrier (system asc_2) does not participate in exchange reactions at 37 °C [8]. In sheep erythrocytes, transport of L-cysteine is also Na^+-independent, and occurs by a low-affinity asc-like system (system C) [9,10]. The occurrence of system asc(C) is not restricted to mammalian erythrocytes. Indeed, asc-like systems have a broad biological distribution. Vadgama and Christensen [11,12] have characterised an asc system in pigeon erythrocytes and we have identified a similar transporter in red cells from the Pacific hagfish (*Eptatretus stoutii*), generally regarded as one of the most primitive living vertebrates [13]. System asc activity has also been found in mammalian exocrine pancreas [14] and may contribute to L-cysteine transport across intestinal basolateral membranes [15].

Inherited deficiencies of erythrocyte amino acid transport occur in both horses and sheep [8,16]. Cells from affected animals lack system asc(C) activity and are consequently predisposed to glutathione deficiency (Fincham, D.A., Mason, D.K. and Young, J.D., unpublished data) [17]. In sheep, this has been shown to lead to a markedly reduced erythrocyte potential life-span [18]. Surprisingly, system C-deficiency also causes the intracellular accumulation of dibasic amino acids [19]. This unexpected observation led to the demonstration that system C has a low, but significant affinity for cationic amino acids, the transporter representing the major route for transport of these amino acids in normal sheep erythrocytes [1]. Similarly, a decreased permeability to dibasic amino acids is observed in system asc-deficient horse erythrocytes [7,8]. The conventional Na^+-dependent ASC system also interacts with dibasic amino acids, particularly L-arginine. In this case, the positively charged guanidinium (or amino) group is considered to occupy the Na^+-binding site on the transporter, allowing dibasic amino acids to act as non-transported, Na^+-independent inhibitors of the system [5,6].

The functional parallels between Na^+-dependent system ASC and Na^+-independent system asc led us to propose that system asc may represent a physiological, genetic or evolutionary variant of ASC [7,8], a suggestion also made previously with respect to the sheep erythrocyte system C [1,10]. According to this hypothesis, system asc is considered to possess a modified or residual Na^+-binding site which permits the transporter to accept and transport cationic amino acids. This view has been challenged by Vadgama and Christensen [11,12]. These authors argue that binding of Na^+ with substrate defines ASC selectivity to an extent that major changes in substrate specificity would result from loss of co-substrate action by Na^+.

In further consideration of the molecular origins of system asc we have undertaken a detailed kinetic analysis of dibasic amino acid transport in horse erythrocytes. Our results establish that systems asc_1 and asc_2 possess specific recognition sites for side chain amino and guanidino groups. Furthermore, we demonstrate that L-arginine causes *cis*- and *trans*-inhibition of system asc_2 in a manner analogous to its interaction with system ASC in pigeon erythrocytes and rabbit reticulocytes.

Methods

Materials. Uniformly ^{14}C-labelled L-amino acids were purchased from Amersham International (Amersham, Bucks., U.K.). Non-radioactive L- and D-amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). When necessary, amino acid solutions were adjusted to the required pH with NaOH or HCl. The organic reagent *n*-dibutylphthalate was obtained from Merck (Darmstadt, F.R.G.).

Animals. Thoroughbred horses were maintained under standard husbandry conditions in the stables of the Royal Hong Kong Jockey Club. Whole blood was obtained by venepuncture and collected into heparinised tubes. Animals were classified as to amino acid transport phenotype on the basis of erythrocyte permeability to L-alanine (0.2 mM, 37 °C) (see Results).

Erythrocytes. Blood was centrifuged at $1000 \times g$ for 10 min and the plasma and buffy coat discarded. Red cells were prepared for transport experiments by washing three times with 20 vol. of an incubation medium containing 150 mM NaCl, 15 mM Mops (pH 7.5 at 37 °C) and 5 mM glucose. Cell suspensions were made to a haematocrit of approx. 20% in this medium. The haemoglobin content of cell suspensions was measured as cyanmethaemoglobin and converted to haematocrit using an experimentally determined conversion factor of 242.5 g haemoglobin/l packed horse erythrocytes.

Amino acid influx. The uptake of amino acids at 37 °C was measured by the cold-start cold-stop method of Young and Ellory [20]. Briefly, 0.2 ml portions of pre-cooled (ice-cold) cell suspension were mixed, in microcentrifuge tubes, with an equal volume of ice-cold medium containing the appropriate concentration of radioactive amino acid. Iso-osmolality at various amino acid concentrations was maintained by adjusting the NaCl composition of the incubation medium. In competition experiments, the inhibitory amino acid and radioactive permeant were added simultaneously. Incubations were initiated by transferring the tubes into a water bath at 37 °C. Incubation times were chosen such that influx at the amino acid concentrations used was linear throughout the experimental period (15 min for L-alanine and L-lysine, 30 min for L-ornithine and 60 min for L-arginine). Uptake was terminated by transferring the tubes back into the ice-bath for a further 15 min. The cells were then pelleted by centrifugation and rapidly washed four times with 1 ml portions of ice-cold medium, using an Eppendorf 5414 microcentrifuge ($15\,000 \times g$ for 10 s). The washed cell pellets were lysed with 0.5 ml of 0.5% (v/v) Triton X-100 in water and deproteinised with 0.5 ml of 5% (w/v) trichloroacetic acid. The precipitate was removed by centrifugation ($15\,000 \times g$ for 2 min) and an aliquot (0.9 ml) of the protein-free supernatant counted for radioactivity by scintillation spectroscopy with appropriate quench correction.

Amino acid efflux. Washed asc_1-type or asc_2-type erythrocytes were incubated at a haematocrit of 30% for 2 or 4 h at 37 °C in incubation medium containing 0.2 or 40 mM L-[U^{14}C]alanine (0.025 μCi/μmol), respectively (see Results). The cells were rapidly washed free of extracellular radioactivity (six times with 10 vol. of ice-cold incubation medium) and finally resuspended in ice-cold medium to a haematocrit of 20%. Radiolabelled L-alanine efflux at 37 °C was measured by mixing 0.5 ml of ice-cold cell suspension with 4.5 ml of pre-warmed incubation medium (control) or medium containing various concentrations of non-radioactive D- or L-amino acids. At pre-determined time intervals (15–60 min), 1 ml portions of cell suspension were removed into ice-cold microcentrifuge tubes containing 0.2 ml of ice-cold *n*-dibutylphthalate and the cells rapidly sedimented below the oil by centrifugation ($15\,000 \times g$ for 20 s). A sample (0.7 ml) of supernatant was immediately removed for scintillation counting. The initial intracellular L-alanine concentration was measured by processing an aliquot of the 'loaded' cell suspension as described for influx. Previous control experiments have established that L-[^{14}C]alanine is not metabolised by horse erythrocytes [8].

Results

L-Alanine influx

Neutral amino acid transport in thoroughbred horse erythrocytes is regulated by three co-dominant allelomorphic genes coding for high-affinity system asc_1, low-affinity system asc_2 and transport-deficiency. These genes are designated h, l and s, respectively. Of the six predicted phenotypes, only five are detected in thoroughbred horses [8]. The three phenotypes selected for the present investigation (groups 1, 3 and 5, Ref. 8) exhibit initial rates of L-alanine uptake in the range 5–15 (group 1), 70–110 (group 3) and 485–625 (group 5) μmol/l cells per h (0.2 mM extracellular concentration, 37 °C) and correspond to genotypes (asc^s, asc^s), (asc^l, asc^l) and (asc^h, asc^s), respectively. Horses of genotype (asc^s,

asc^{s}) are deficient with respect to erythrocyte asc activity (transport-deficient type) whereas the two transport-positive genotypes exhibit system asc_2 and system asc_1 activity, respectively.

Representative concentration-dependence curves for L-alanine uptake by these three cell types over both low- and high-concentration ranges (0.05–5 mM and 2.5–100 mM, 37 °C) are presented in Figs. 1A and B. In agreement with previous studies [7,8], L-alanine uptake by transport-deficient erythrocytes was slow and linear with respect to concentration (3.6 mmol/l cells per h at 100 mM extracellular L-alanine). The two transport-positive cell types exhibited additional saturable components of L-alanine influx corresponding to system asc-mediated uptake. Kinetic constants for this uptake, calculated by linear regression analysis of plots of s/v against s (using values of v corrected for the non-saturable component of L-alanine influx as measured in transport-deficient cells), are presented in Table I. Apparent K_m values were 0.35 mM for the asc_1-type cells and 14 mM for the asc_2-type cells, a difference of 40-fold. It has been established previously that systems asc_1 and asc_2 do not require Na^+ for activity [8].

TABLE I

KINETIC CONSTANTS FOR NEUTRAL AND DIBASIC AMINO ACID UPTAKE BY TRANSPORT-POSITIVE HORSE ERYTHROCYTES

Initial rates of (U-^{14}C)-labelled L-amino acid uptake were measured at 37 °C. The kinetic parameters were determined as described in the text.

Amino acid	System asc_1		System asc_2	
	K_m (mM)	V_{max} (mmol/l cells per h)	K_m (mM)	V_{max} (mmol/l cells per h)
L-Alanine	0.35	1.7	14	6.9
L-Arginine	27	0.54	14	0.09
L-Ornithine	27	2.2	> 100	–
L-Lysine	9	1.7	≈ 90	–

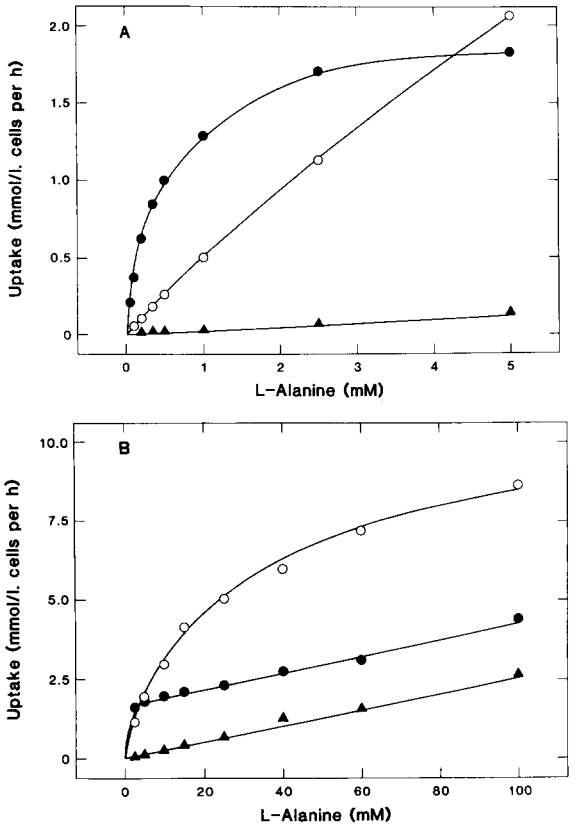

Fig. 1. Concentration-dependence of L-alanine uptake by transport-deficient and transport-positive horse erythrocytes. Initial rates of L-[U-^{14}C]alanine uptake (extracellular concentrations 0.05–5 mM (A) and 2.5–100 mM (B)) were measured at 37 °C in NaCl medium. Transport-deficient cells (▲), asc_1-type cells (●) and asc_2-type cells (○). Values are the means of triplicate estimates from the same experiment.

Dibasic amino acid influx

Fig. 2 compares the concentration-dependence of L-ornithine (A), L-lysine (B), and L-arginine (C) uptake by transport-deficient and transport-positive asc_1-type and asc_2-type erythrocytes from the same horses used in the experiment shown in Fig. 1. The extracellular concentration range was 2.5–100 mM for each amino acid. Uptake of all three dibasic amino acids by transport-deficient cells was slower than for L-alanine and linear with respect to concentration, L-arginine giving the lowest uptake rate (2.03, 0.46 and 0.27 mmol/l cells per h at 100 mM extracellular L-ornithine, L-lysine and L-arginine, respectively). These results correspond well with previous determinations of dibasic amino acid uptake in transport-deficient cells measured at 0.2 mM extracellular concentration [7,8] and indicate the absence of a high-affinity dibasic amino acid transporter (system y^+) in

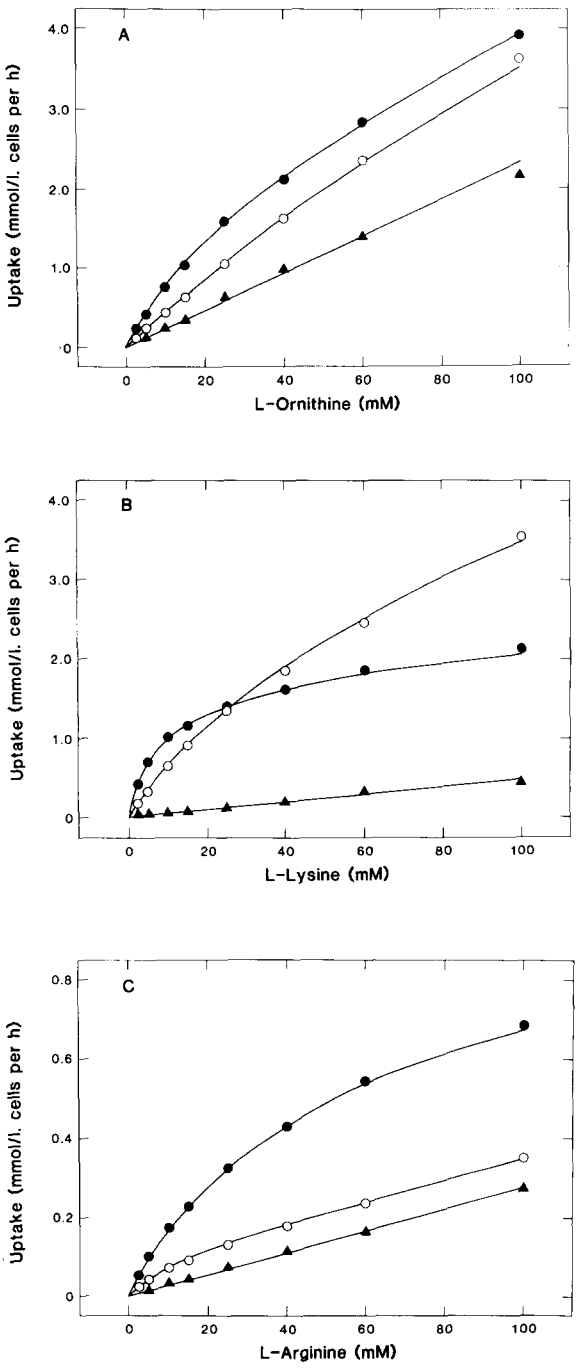

Fig. 2. Concentration-dependence of dibasic amino acid uptake by transport-deficient and transport-positive horse erythrocytes. Initial rates of (U-^{14}C)-labelled L-ornithine (A), L-lysine (B) an L-arginine (C) uptake (2.5–100 mM) were measured at 37 °C in NaCl medium. Transport-deficient cells (▲), asc_1-type cells (●), and asc_2-type cells (○). Values are the means of triplicate estimates from the same experiment.

erythrocytes of this species. Similarly, previous kinetic studies of neutral amino acid transport have established that horse erythrocytes lack amino acid transport systems L and A [7,8], potential low-affinity routes for dibasic amino acid transport [3,4]. Small neutral (and acidic) amino acids can also be accepted as accidental substrates for the Band 3 anion-exchange transporter [21,22]. However, L-lysine uptake by group 1 cells was unaffected by 20 μM SITS, suggesting that this pathway does not contribute towards dibasic amino acid uptake by horse erythrocytes (data not shown).

In contrast, transport-positive cells exhibited additional saturable components of dibasic amino acid transport. Kinetic constants for this uptake (again calculated using values of v corrected for the non-saturable component of uptake in transport-deficient cells) are presented in Table I. It can be seen that in cells with the low-affinity asc_2 transporter, the saturable component of L-arginine uptake had the same apparent K_m value (14 mM) as L-alanine, an optimal substrate of the system, but a 77-fold lower V_{max}. By contrast, the apparent K_m values for L-lysine and L-ornithine transport were very much higher ($\approx$ 90 and $>$ 100 mM, respectively). V_{max} values for these latter two amino acids could not be determined with any accuracy, but were most likely in the same range as the V_{max} for L-alanine uptake (6.9 mmol/l cells per h).

Kinetic constants calculated for saturable dibasic amino acid transport in cells with the high-affinity asc_1 transporter were markedly different to those for asc_2-type erythrocytes (see also Table I). Thus, the apparent K_m for L-arginine uptake (27 mM) was approximately 2-fold higher than that measured for asc_2-type cells and almost 80-fold higher than the corresponding apparent K_m for L-alanine influx in the same erythrocytes. Also, the difference in V_{max} for L-arginine and L-alanine uptake by asc_1-type cells (3.1-fold) was not as marked as that for asc_2-type erythrocytes. V_{max} values for L-ornithine and L-lysine uptake by asc_1-type cells were similar to the V_{max} for L-alanine transport. However, the apparent affinities for mediated transport of these two dibasic amino acids were 77- and 25-fold lower than for L-alanine, respectively, L-ornithine having the same apparent

K_m value as L-arginine. Thus, kinetically, all three dibasic amino acids seemed to function as conventional low-affinity substrates for the asc_1 transporter. Calculated V_{max} : K_m ratios for L-lysine, L-ornithine and L-arginine were 0.18, 0.08 and 0.02, respectively, compared with 4.8 for L-alanine. The observed preference for L-lysine over L-ornithine contrasts with the specificity which system asc_1 exhibits for the larger neutral amino acids where transport activity decreases with increasing side chain length.

Effects of dibasic amino acids on L-alanine influx

To confirm that dibasic and neutral amino acids share common routes for transport across the horse erythrocyte membrane and to investigate transporter interactions with dibasic amino acids in more detail, saturable L-alanine uptake by the two transport-positive cell types was measured at approximate K_m concentrations (0.3 mM for system asc_1 and 15 mM for system asc_2) in the presence of various concentrations (0–100 mM) of a series of different dibasic amino acids with either a side chain amino group (L-2,4-diaminobutyrate, L-ornithine and L-lysine) or a guanidino side chain group (L-2-amino-4-guanidinobutyrate, L-arginine and L-homoarginine). Results are presented in Fig. 3, the data corrected for the contribution of the non-saturable component of amino acid uptake (which was not inhibited by dibasic amino acids) using L-alanine uptake values measured in transport-deficient cells under the same experimental conditions. The most effective inhibitors of L-alanine transport by system asc_1 were L-2-amino-4-guanidinobutyrate and L-lysine, the concentrations required to cause 50% inhibition of uptake being 9 and 12 mM, respectively (Figs. 3A and B). L-Arginine was considerably less effective than L-2-amino-4-guanidinobutyrate (50% inhibition at 55 mM extracellular concentration) and increasing the length of the side chain (L-homoarginine) further decreased the amount of inhibition observed (Fig. 3A). The order of effectiveness for dibasic amino acids possessing a side chain amino group decreased with decreasing side chain length (L-lysine > L-ornithine > L-2,4-diaminobutyrate) (Fig. 3B).

L-Arginine was the most effective inhibitor of L-alanine transport via system asc_2 (50% inhibition at 14 mM extracellular concentration) (Fig. 3C). Either an increase (L-homoarginine) or a decrease (L-2-amino-4-guanidinobutyrate) in the length of the amino acid side chain resulted in a loss of inhibition compared with L-arginine. Dibasic amino acids containing an amino group in the side chain were relatively poor inhibitors of system asc_2 (L-lysine > L-ornithine > L-2,4-diaminobutyrate) (Fig. 3D).

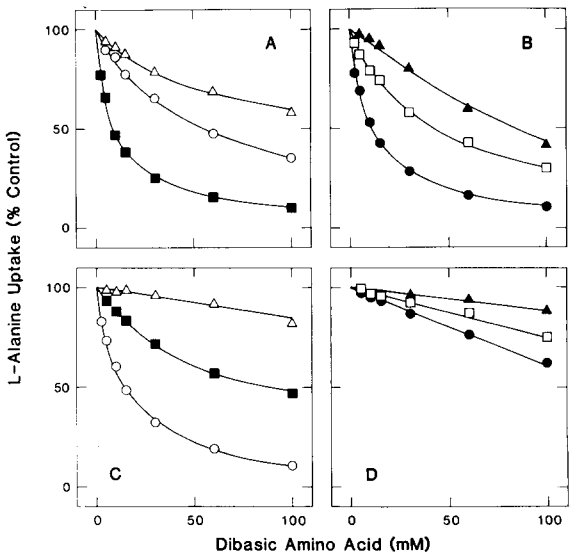

Fig. 3. Effects of dibasic amino acids on L-alanine uptake by transport-positive horse erythrocytes. Initial rates of L-[U-^{14}C]alanine transport (37 °C) were measured at extracellular concentrations of 0.3 mM (A and B) and 15 mM (C and D) in asc_1-type and asc_2-type cells, respectively. Non-radioactive dibasic amino acids were present at extracellular concentrations in the range 2.5–100 mM. Data for both cell types are corrected for L-alanine uptake by transport-deficient cells under the same experimental conditions and expressed as a percentage of the uninhibited control. L-2-Amino-4-guanidinobutyrate (■), L-arginine (○), L-homoarginine (△) (A and C); L-2,4-diaminobutyrate (▲), L-ornithine (□), L-lysine (●) (B and D). Values are the means of triplicate estimates from the same experiment.

In another experiment, uptake of L-alanine (3–30 mM) by system asc_2 was measured in the presence of varying concentrations of L-arginine (20–100 mM). Dixon [23] analysis of the data (corrected for the non-saturable component of transport measured in transport-deficient cells) confirmed that inhibition was competitive, with an apparent K_i value of 6.8 mM (Fig. 4). Substitution of Na^+ in the incubation medium by K^+ had

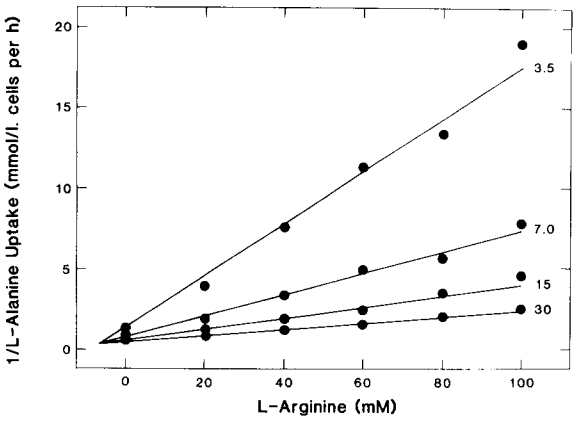

Fig. 4. Effect of L-arginine on L-alanine uptake by transport-positive horse erythrocytes. Initial rates of L-[U-^{14}C]alanine transport (37 °C) by asc_2-type cells were measured at extracellular concentrations in the range 3.5–30 mM (indicated to right of figure). Non-radioactive L-arginine was present at extracellular concentrations in the range 20–100 mM. Data are corrected for the non-saturable component of L-alanine uptake measured in transport-deficient cells. Values are the means of triplicate estimates from the same experiment.

no effect on L-arginine inhibition of system asc_2 activity (data not shown).

Effects of L-alanine on dibasic amino acid influx

Complementary cross-inhibition experiments to those shown in Fig. 3 are presented in Fig. 5, where L-arginine, L-lysine and L-ornithine uptake were measured at an extracellular concentration of 0.2 mM in the presence of various concentrations of L-alanine (0–10 mM for cells with the high-affinity asc_1 transporter and 0–100 mM for cells possessing the low-affinity asc_2 system). As in the previous series of experiments, the contribution of non-saturable dibasic amino acid uptake (as measured in transport-deficient cells) was subtracted from the transport-positive cell data. Control experiments confirmed that L-alanine had no effect on dibasic amino acid uptake in transport-deficient erythrocytes (data not shown). The results demonstrate that carrier-mediated dibasic amino acid transport can be inhibited by L-alanine. The concentrations of L-alanine required to inhibit L-ornithine, L-lysine and L-arginine uptake by 50% were 0.4, 0.3 and 0.8 mM, respectively for asc_1-type cells and 10, 10 and 11 mM, respectively, for asc_2-type cells.

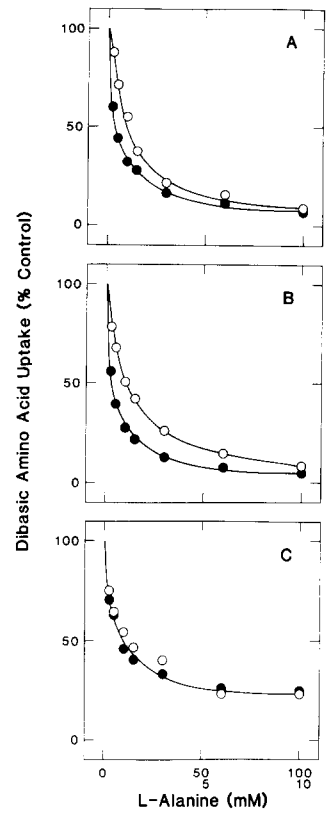

Fig. 5. Effects of L-alanine on dibasic amino acid uptake by transport-positive horse erythrocytes. Initial rates of (U-^{14}C)-labelled L-ornithine (A), L-lysine (B) and L-arginine (C) transport (37 °C) were measured at an extracellular concentration of 0.2 mM in asc_1-type cells (●) and asc_2-type cells (○). Non-radioactive L-alanine was present at extracellular concentrations in the range of 0.25–10 mM for asc_1-type cells and 2.5–100 mM for asc_2-type cells. Data are corrected for dibasic amino acid uptake by transport-deficient cells under the same experimental conditions and expressed as a percentage of the uninhibited control. Values are the means of triplicate estimates from the same experiment.

These results provide further strong evidence that neutral (L-alanine) and dibasic amino acids share common routes for transport across the horse erythrocyte membrane.

Effects of extracellular neutral and dibasic amino acids on L-alanine efflux

In a final series of experiments, we compared the effects of different dibasic and neutral amino acids on L-alanine efflux from asc_1-type and asc_2-type erythrocytes. In Figs. 6A and B we show

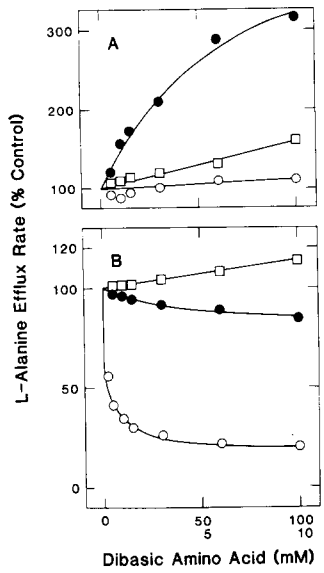

Fig. 6. Effects of extracellular dibasic amino acids on L-alanine efflux from transport-positive horse erythrocytes. Cells possessing system asc_1 (A) and system asc_2 (B) transport activity were loaded with L-[U-^{14}C]alanine to intracellular concentrations of 0.18 and 10.5 mmol/l cells, respectively. Initial rates of L-[U-^{14}C]alanine efflux were measured at 37 °C in the presence of various concentrations of extracellular non-radioactive L-arginine (○), L-ornithine (□) and L-lysine (●) (0–10 mM, A; 0–100 mM, B).

initial rates of L-alanine efflux measured at 37 °C as a function of extracellular L-ornithine, L-lysine and L-arginine concentration (0–10 mM for asc_1-type cells, and 0–100 mM for asc_2-type cells). L-Ornithine caused moderate stimulation of L-alanine efflux from cells possessing system asc_1 activity (Fig. 6A). This stimulation was linear with respect to L-ornithine concentration, reaching a value 65% above control (efflux measured in the absence of extracellular amino acid) at 10 mM L-ornithine. In contrast, the strong stimulation of L-alanine efflux by L-lysine was saturable with respect to extracellular dibasic amino acid concentration, reaching a value 200% above control at 10 mM extracellular L-lysine. L-Arginine also caused a slight increase in L-alanine efflux from cells with system asc_1 transport activity. The relative abilities of these amino acids to stimulate L-alanine efflux from asc_1 type cells is consistent with the order of preference shown for dibasic amino acid uptake by system asc_1 (Fig. 2, Table I).

In marked contrast, extracellular L-lysine and L-arginine caused inhibition of L-alanine efflux from cells with system asc_2 activity. In the case of L-lysine, the inhibition was weak (16% inhibition

TABLE II

EFFECTS OF AMINO ACIDS ON L-ALANINE EFFLUX FROM TRANSPORT-POSITIVE HORSE ERYTHROCYTES

Initial intracellular L-[U-^{14}C]alanine concentrations were 0.22 mM for asc_1-type cells and 15.5 mM for asc_2-type cells. Results are expressed as a percentage of the control efflux rate measured at 37 °C in the absence of extracellular amino acid. –, not tested.

Amino acid	System asc_1		System asc_2			
	Isomer		Isomer			
	L (5 mM)	D (5 mM)	L		D	
			10 mM	50 mM	10 mM	50 mM
Alanine	430	174	99	96	100	107
Leucine	94	107	85	43	103	–
Isoleucine	100	105	90	47	105	–
Norleucine	106	103	97	85	103	–
Methionine	103	108	103	92	106	–
Phenylalanine	97	101	94	55	104	–
2,4-Diaminobutyrate	130	–	102	113	–	–
Ornithine	176	104	102	102	101	101
Lysine	220	106	97	87	100	100
2-Amino-4-guanidinobutyrate	249	–	97	–	–	–
Arginine	117	99	37	21	98	80
Homoarginine	96	–	98	98	–	–

at 100 mM dibasic amino acid), whereas L-arginine was a highly effective inhibitor of L-alanine efflux, 50% inhibition occurring at 3 mM extracellular amino acid (Fig. 6B). The concentration of L-arginine required to induce half the maximum inhibitory response was 0.5 mM. In this cell type, L-ornithine was able to stimulate L-alanine efflux, the response being linear with respect to concentration (13% stimulation at 100 mM extracellular amino acid) (Fig. 6B).

As shown in Table II, dibasic amino acid stimulation of L-alanine efflux from asc_1-type cells correlated well with the influx inhibition data presented in Figs. 3A and B, and was stereospecific. For example, at the extracellular amino acid concentration used in this experiment (5 mM), L-lysine caused a 120% increase in the rate of L-alanine efflux compared with 7% for D-lysine and 330% for L-alanine. In contrast, a representative series of large neutral amino acids caused minimal stimulation of L-alanine efflux via system asc_1. The results presented in Table II also demonstrate that L-arginine was a more effective inhibitor of L-alanine efflux from asc_2-type cells than D-arginine. In agreement with previous studies [8], extracellular L-alanine had no effect on radiolabelled L-alanine efflux from this cell type. High concentrations of large neutral amino acids were, however, moderately inhibitory.

Discussion

Dibasic amino acid uptake by horse erythrocytes deficient in system asc_1 and asc_2 activity may well represent simple diffusion of amino acid across the lipid bilayer. Equivalent non-saturable components of dibasic amino acid uptake are also apparent in transport-positive asc_1-type and asc_2-type horse erythrocytes, the asc transporters representing the only mediated routes of dibasic amino acid transport in erythrocytes of this species. This contrasts markedly with the complex amino acid transport profiles of human and avian erythrocytes where several different amino acid transporters are present in the one cell type [1–6]. The data presented in Fig. 3 and Table I establish that the relative abilities of different dibasic amino acids to inhibit L-alanine uptake by the two transport-positive cell types correspond well with their apparent K_m values for transport, while in Table II we show that the interactions of dibasic amino acids with system asc_1 are stereospecific and that the transporter exhibits a strong preference for dibasic amino acids compared with neutral amino acids of similar size. This latter aspect of selectivity is also apparent from the inability of system asc_1 to transport large neutral amino acids (see Table I, Ref. 8).

We interpret these results as evidence that system asc_1 has a specific recognition site which enhances interaction with the positively-charged group present on the side chain of dibasic amino acids. By this means the transporter exhibits a dual selectivity for neutral amino acids of intermediate size and cationic amino acids, effectively combining the transport functions of systems ASC and y^+. The location of this recognition site (as inferred from the optimal position of the side chain positive charge) corresponds with that expected for the Na^+-binding site on system ASC [3–6]. L-Lysine, one of the most effective dibasic amino acid permeants on system asc_1, had an apparent K_m value of 9 mM compared with 0.35 mM for L-alanine.

Further evidence for the existence of such a recognition site is provided by the dramatic *cis*- and *trans*-inhibitory effects of L-arginine on system asc_2. Kinetically, this amino acid was found to have the same apparent K_m value for influx as L-alanine (14 mM), an optimal asc substrate, but an almost 80-fold lower V_{max} (Table I). These results indicate that L-arginine has a high relative affinity for the asc_2 transporter but that the 'mobility' of the L-arginine-carrier complex is very low. In effect, therefore, L-arginine functions as a non-transported inhibitor of system asc_2. D-Arginine caused substantially less inhibition of system asc_2 activity than L-arginine, but was more effective than either L-ornithine or L-lysine (Table II) (see also Table I, Ref. 5). The ability of L-arginine to cause *trans*-inhibition of the asc_2 transporter has potential implications for normal amino acid transport function in horse erythrocytes. Red cells from most horses exhibit an inherited deficiency of the enzyme arginase, resulting in the accumulation of millimolar concentrations of L-arginine within the cells [24]. Thus, intracellular L-arginine may act physiologically as an endoge-

nous inhibitor of system asc_2 activity, contrasting with the ability of intracellular amino acids to enhance amino acid uptake via system asc_1 [7,8]. Amino acid transport systems A and N are also subject to *trans*-inhibition by intracellular amino acids [25].

Our observations with horse erythrocytes show remarkable parallels with the ability of L-arginine to inhibit Na^+-dependent L-serine transport by the pigeon erythrocyte, rabbit reticulocyte and Ehrlich ascites ASC systems. In particular, inhibition of system ASC activity by L-arginine is not associated with detectable L-arginine translocation [3–6]. Furthermore, this inhibition is only partially stereospecific, but is selective for L-arginine versus L-ornithine and L-lysine; apparent K_i values for the latter two amino acids are five times higher than that for D-arginine, which is in turn twice that for L-arginine (8 mM) [5]. A further point of similarity between the ASC and asc_2 systems is the reported ability of non-transported neutral amino acids with bulky side chains to exert Na^+-independent inhibition of ASC activity [26]. It was considered likely that apolar binding of these amino acids at the transport site obstructed Na^+-binding, hence preventing transport. Data presented in Table II demonstrate that high concentrations of large neutral amino acids significantly inhibit L-alanine efflux by system asc_2. As is the case for system asc_1, large neutral amino acids are not significant asc_2 substrates (Table I, Ref. 8).

As mentioned in the Introduction, pigeon erythrocytes also possess an asc system, discriminated from other amino acid transporters present by its pH sensitivity and by cross-competition experiments in Na^+-free medium [11]. Thus, the pigeon asc system was found to have a similar substrate specificity to the horse and sheep transporters, exhibiting a selectivity for L-serine, L-alanine, L-threonine and L-valine. However, it is not known whether the avian asc system resembles systems asc_1 or asc_2 (C) kinetically. Vadgama and Christensen [11] do not consider system asc to be a Na^+-independent variant of ASC. They contend that Na^+ plays a critical role in determining the amino acid selectivity of system ASC, such that the substrate specificity would be changed dramatically, if indeed any amino acid binding survived at all, in the absence of Na^+ or Na^+ binding. The basis of this argument concerns evidence that Na^+ and amino acid bind in juxtaposition, such that a hydroxyl or other polar group on the side chain, if suitably located (optimally on carbon-4, and *trans* with respect to the α-carboxyl group), can participate in a direct bond between the two co-substrates [5,6]. Whilst this interaction may conceivably be important for atypical substrates such as L-hydroxyproline [5], it is unlikely to be of major significance for other, more representative ASC permeants. In particular, direct co-substrate binding would not be expected to be important in defining ASC interactions with amino acids having apolar side chains, i.e., L-alanine and L-α-amino-*n*-butyrate. Nevertheless, L-alanine has essentially identical apparent K_m and V_{max} values for uptake by the pigeon erythrocyte ASC system as L-serine and L-cysteine [27], and L-α-amino-*n*-butyrate is an excellent substrate for the human erythrocyte ASC system [2]. As might be expected, the hydroxyprolines do not exhibit significant interactions with the horse erythrocyte asc systems (Fincham, D.A., Mason, D.K. and Young, J.D., unpublished data).

Some of the functional similarities between systems ASC and asc could be the product of convergent evolution of unrelated transporter polypeptides. However, our present observations, particularly those regarding the interaction of L-arginine with the horse erythrocyte asc_2 system, persuade us that there may be a more direct structural relationship between ASC and the mammalian asc (C) systems, the two classes of amino acid transporter possessing equivalent recognition sites for amino acid side chain cationic groupings. In the case of system ASC, Thomas and Christensen [5,6] have identified this site as the site of Na^+ binding. The ASC site also accepts free guanidinium$^+$ [5]. The corresponding site on system asc does not exhibit a significant affinity for Na^+ (L-arginine inhibition of system asc_2 is independent of Na^+ concentration), but does interact with both guanidinium$^+$ (Fincham, D.A., Mason, D.K. and Young, J.D., unpublished data) and with the Na^+ site inhibitor, harmaline [28].

The small amount of kinetic data which is available indicates that amino acid and Na^+ bind to the ASC transporter in random order [6]. This

type of mechanism implies that the transporter exhibits significant affinity for amino acid in the absence of Na^+ (see also above). In the simplest possible case, asc-like activity, such as that found in the pigeon erythrocyte, could arise as a form of 'slippage' [29] generated by reduction or removal of the activation energy barrier(s) normally preventing translocation of the binary neutral (or dibasic) amino acid-carrier complex. True Na^+-independent transport of the type found in horse and sheep erythrocytes would require modification of the Na^+-binding site so as to reduce the transporter's affinity for Na^+. Such functional changes, including system asc's tolerance for L-valine [7,8,11,12] could arise from relatively minor alterations in transporter structure.

In conclusion, the present series of experiments provide further evidence of functional and structural similarities between Na^+-independent amino acid transport systems asc_1 and asc_2 from horse erythrocytes and Na^+-dependent system ASC. It remains to be established whether system ASC and the mammalian asc(C) systems represent different physiological expressions of the same transporter or whether the two transport activities are mediated by separate, but perhaps homologous proteins.

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