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Testing the Hypothesis that System y^+L Accounts for High- and Low-Transport Phenotypes in Chicken Erythrocytes Using L-Leucine as Substrate

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Abstract. Experiments were carried out to test the hypothesis that system y^+L accounts for the high (HT) and low (LT) amino-acid transport phenotypes in chicken erythrocytes and to explain the different effect of selective breeding on lysine and leucine fluxes. L-Leucine transport was characterized in individuals which had been separated into two groups (HT and LT) according to their capacity to transport L-lysine across the erythrocyte membrane. Whereas lysine influx (1 μM) in the two groups differed by 32-fold (HT/LT), leucine influx was not significantly different. Average rates (nmol/ L cells/ min) were: 227 (HT) and 7.0 (LT) for L-lysine, and 8.9 (HT) and 7.1 (LT) for L-leucine. The differential ability of L-lysine and L-leucine fluxes to discriminate between the HT and LT phenotypes was shown to be consistent with the interactions of these substrates with system y^+L and to vary depending on the conditions of the assay. It is shown that the two phenotypes can be clearly discriminated by measuring L-leucine influx in the presence of Li^+. These results support the hypothesis that the HT and LT phenotypes reflect alterations in the function of system y^+L and illustrate that the choice of the appropriate substrate and medium composition must be carefully considered when investigating the consequences of either experimental or natural alterations of broad-scope transporters.

Key words: Leucine — Amino acids — y^+L — Transport — Erythrocytes

Introduction

Early studies revealed important individual differences in the capacity of chicken to transport amino acids across the erythrocyte membrane and showed that these differences could be emphasized by genetic selection. Although heterogeneity was seen to affect both L-lysine and L-leucine transport, the changes induced by selective breeding were found to be larger for lysine than for leucine. Further investigations, carried out at the time to explain these findings, were not successful (Lerner et al., 1978; Somes, Smagula & Lerner, 1981; Lerner et al., 1982).

Later, it was proposed that the heterogeneity in lysine transport reflects differences in the function of system y^+L, a broad-spectrum and cation-modulated amino-acid transporter that belongs to the family of heteromeric transporters (Vargas & Devés, 2001). Two phenotypes exhibiting either low (LT) or high (HT) rates of L-lysine transport were described and the average rates of entry of L-lysine (1 μM) in the two groups were shown to differ by a factor of approximately 40-fold. Correspondence between these activities and system y^+L is supported by observations on specificity, transport mechanism and sensitivity towards sulfydryl reagents. In both phenotypes, the transporter is able to recognize and translocate cationic and neutral amino acids in the presence of Na^+ (or Li^+) but in the presence of K^+, it becomes selective for cationic amino acids. Lysine transport in both LT and HT phenotypes reflects the operation of a tightly coupled exchanger, as it is the case for most members of the heteromeric family of transporters (Angelo & Devés, 1994; Chillarón et al., 1996; Verrey et al., 1999) and is inactivated by p-chloromercuribenzene sulfonate (PCMBS), but resistant to N-ethylmaleimide (NEM). In spite of this general similarity between the lysine transport activity in the red cells of HT and LT individuals, the specificities are not identical, as deduced from competition and *trans*-acceleration experiments. The transport specificity in LT erythrocytes resembles that of system y^+L, originally described in human erythrocytes, showing comparable affinities for leucine and lysine in the

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presence of Na^+ (Devés, Chávez & Boyd, 1992; Devés, Angelo & Chávez, 1993; Kanai et al, 2000; reviewed in Devés & Boyd, 1998 and Verrey et al., 2004). The HT activity exhibits some singular attributes, the most distinctive of them being its relatively low affinity for leucine (the half-saturation constant for leucine is 10 times larger than that of the LT group); nevertheless, it interacts strongly with other neutral amino acids (glutamine, methionine), as is the case for the LT transporter. In the presence of Li^+, the strength of leucine binding to the HT transporter is dramatically increased.

In this study, an analysis of leucine transport in HT and LT erythrocytes was carried out to further test the hypothesis that system y^+L accounts for the HT and LT phenotypes. In addition, the study aimed to understand the cause of the apparent anomalies observed in the selective breeding experiments with regard to the magnitude of lysine and leucine fluxes. The results are in agreement with the hypothesis that the high- and low-transport phenotypes reflect alterations in the function of system y^+L and the differences found in the magnitude of the L-leucine and L-lysine fluxes in HT and LT erythrocytes are explained.

Materials and Methods

Materials

L-[^{14}C]-Lysine and L-[^{14}C]-leucine (approximately 300 mCi/mmol) were purchased from ARC (American Radiolabeled Chemicals) and unlabeled amino acids, chloramphenicol, N-ethylmaleimide (NEM), and p-chloromercuribenzene sulfonate (pCMBS), from Sigma. All other chemicals were of commercial reagent grade. Healthy adult male Leghorn chickens were obtained from a commercial farm and fed on a regular poultry diet.

Preparation of Cells

Fresh blood was obtained by cardiac puncture (under ketamine anesthesia) using heparin as an anticoagulant. The blood was spun and the plasma, buffy coat and upper layer of cells (including white cells and platelets) were removed by aspiration. Erythrocytes were then washed with "Na^+ medium" containing (in mM): 133 NaCl, 3 KCl, 1.2 $MgSO_4$, 3 NaH_2PO_4, and 6 Na_2HPO_4 (pH 7.4) and packed by centrifugation in an isotonic medium (the composition depending on the particular experiment). Other isotonic solutions used were (in mM) 1.2 $MgSO_4$, 3 KH_2PO_4, 6 K_2HPO_4 plus 133 KCl (K^+ medium) or 133 lithium chloride (Li^+ medium). Unless indicated, experiments were performed in Na^+ medium.

Measurement of Entry Rates

Entry was followed as previously described (Vargas & Devés, 2001). Briefly, packed cells were added to a solution containing L-[^{14}C]leucine or L-[^{14}C]lysine in isotonic saline (10% hematocrit, 39°C). The composition of the external medium varied depending on the experiment. Three samples of the suspension were withdrawn at intervals and placed in tubes containing dibutylphthalate. After centrifugation, the cells sedimented below the organic layer. The aqueous supernatant was taken off by aspiration and the walls of the tube were thoroughly washed. Following the aspiration of dibutylphthalate, the cells were disrupted by addition of 5% trichloroacetic acid. The suspension was spun (1 min at $11{,}000 \times g$) and the radioactivity in the supernatant determined by scintillation counting.

Initial uptake rates were calculated by linear regression from the relationship of the intracellular radioactivity and time. All determinations were performed in duplicate runs, and therefore initial rates were generally estimated from six time points. When deviation from initial rates was evident, the measurements performed at longer times were not considered in the analysis. Mediated transport of lysine and leucine was estimated by subtraction of the transport rates measured in presence of 2 mM of unlabeled lysine or 10 mM of unlabeled leucine from the total flux, respectively.

Measurement of Exit Rates

Exit was followed as previously described (Angelo & Devés, 1994). Washed cells were incubated in Na^+ medium (50% hematocrit), containing L-[^{14}C]leucine or L-[^{14}C]lysine (10 μM) and chloramphenicol (0.02%) and incubated at 39°C for 3 h, except when lysine exit was measured in HT erythrocytes. In this case, cells were loaded for 30 min. After this period, the cells were separated from the external medium by centrifugation and repeated washes with ice-cold buffer. Aliquots of loaded cells were added to the assay medium (10% hematocrit, 39°C) and three samples were withdrawn at intervals and rapidly centrifuged in tubes containing dibutylphthalate. The cells sedimented below the organic layer and the radioactivity in the supernatant was determined by scintillation counting. All determinations were done in duplicate runs.

Determination of the Half-Saturation Constants (K_i)

Inhibition by unlabeled amino acids was analyzed according to the following equation:

$$v_o/v = 1 + [A]/K_{iA} \quad (1)$$

where v_o and v are the rates of entry of labeled lysine in the absence or presence of unlabeled amino acids, respectively. $[A]$ is the concentration of unlabeled amino acid and K_{iA} the half-saturation (or inhibition) constant for analogue A. It is assumed that the external lysine concentration is very low ([lysine] $\ll K_m$). Inhibition constants were calculated by non-linear regression analysis of the data. This is a valid assumption in the present study because the substrate concentration used was 1 μM.

Results

Individuals were separated into two groups (HT and LT) according to their capacity to transport L-lysine across the erythrocyte membrane and experiments were carried out to characterize L-leucine transport in these groups. The average value of the rate of entry of lysine (1 μM) into erythrocytes in each of the two

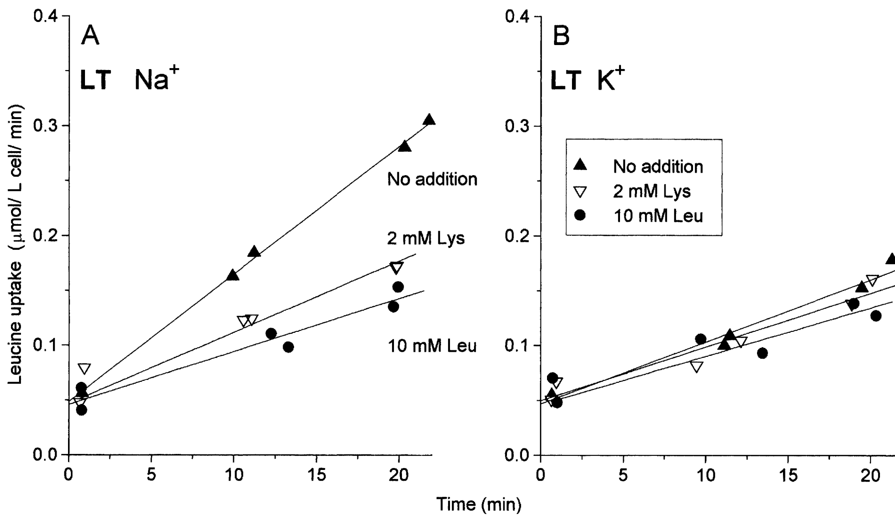

Fig. 1. Uptake of L-leucine in erythrocytes exhibiting low lysine transport (LT). Influx of L-[^{14}C]-leucine was measured in the absence (▲) or presence of 2 mM unlabelled lysine (▽) or 10 mM unlabelled leucine (●). The external medium contained Na^+ (*A*) or K^+ (*B*) as the principal cation. The concentration of L-leucine was 1 μM. Erythrocytes were obtained from one individual representative of the LT phenotype.

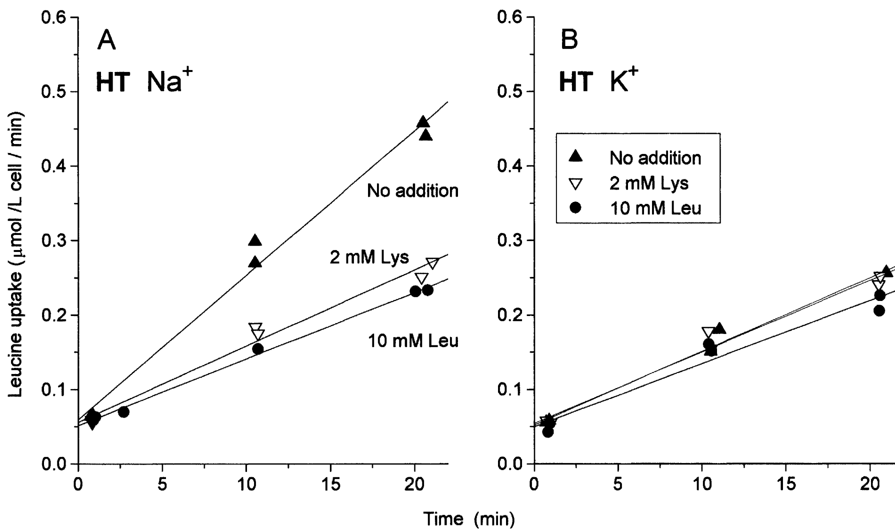

Fig. 2. Uptake of L-leucine in erythrocytes exhibiting high lysine transport (HT). Influx of L-[^{14}C]-leucine was measured in the absence (▲) or presence of 2 mM unlabelled lysine (▽) or 10 mM unlabelled leucine (●). The external medium contained Na^+ (*A*) or K^+ (*B*) as the principal cation. The concentration of L-leucine was 1 μM. Erythrocytes were obtained from one individual representative of the HT phenotype.

groups was (nmol/ L cells/ min) 227 ± 26.4 (n = 7) in HT and 7.0 ± 1.1 (n = 9) in LT.

Mediated uptake of L-leucine (1 μM) into chicken erythrocytes was found to be partially inhibited by unlabelled L-lysine added to the external medium at a concentration that is sufficient to saturate system y$^+$L Representative experiments carried out in LT and HT erythrocytes, in the presence of Na^+, are shown in Fig. 1*A* and Fig. 2*A*, respectively. The lysine-sensitive leucine flux was not observed when Na^+ in the medium was replaced by K^+ (Fig. 1*B* and Fig. 2*B*). The fraction of the flux that can be ascribed to system y$^+$L (lysine-sensitive and Na^+-dependent) was on average 76% in HT erythrocytes and 65% in LT erythrocytes. The behavior of leucine in exit experiments was not consistent with the functional properties of system y$^+$L, which has been shown to operate as an efficient amino-acid exchanger. A comparison of the exit of L-[^{14}C]lysine and L-[^{14}C]leucine into Na^+ medium, with or without

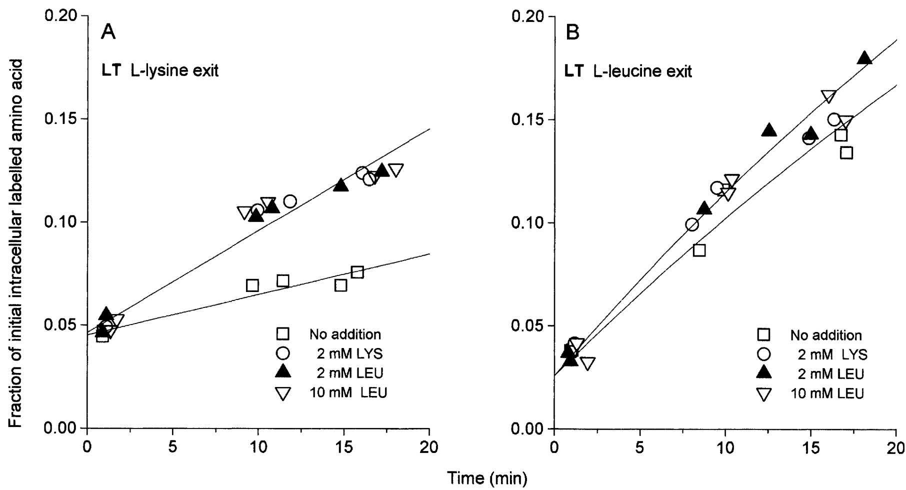

Fig. 3. Exit of L-[^{14}C]lysine (*A*) and L-[^{14}C]leucine (*B*) in LT erythrocytes. The external medium contained either 2 mM lysine (○), 2 mM leucine (▲), 10 mM leucine (▽) or no addition (□). The initial concentration of lysine and leucine in the intracellular medium was 4.3 μM and 7.8 μM, respectively. The initial rates (μM/ min) in the presence of 2 mM lysine in the external medium were 0.021 ± 0.002 (lysine exit) and 0.057 ± 0.004 (leucine exit). Erythrocytes were obtained from one individual representative of the LT phenorype. The external medium contained Na^+ as the principal cation.

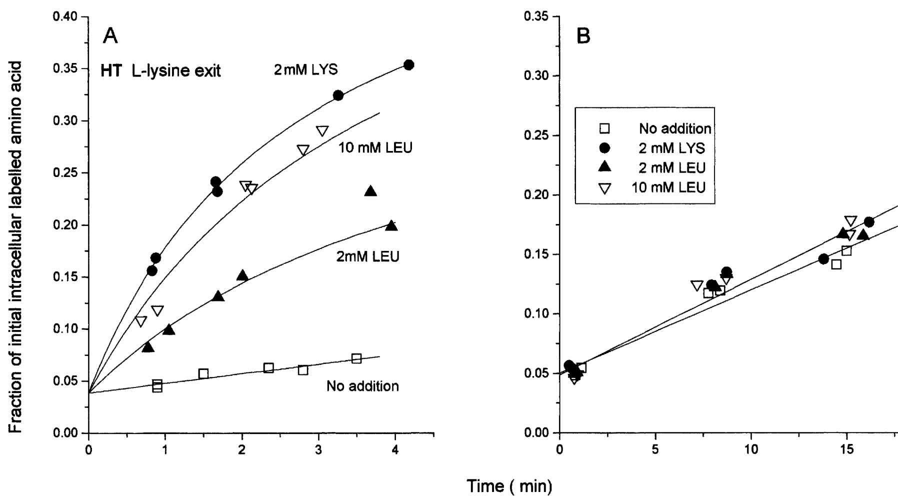

Fig. 4. Exit of L-[^{14}C]lysine (*A*) and L-[^{14}C]leucine (*B*) from HT erythrocytes. The external medium contained either 2 mM lysine (●), 2 mM leucine (▲) or 10 mM leucine (▽) or no addition (□). The initial concentration of lysine and leucine in the intracellular medium was 4.4 μM and 5.0 μM, respectively. The initial rates (μM/ min) in the presence of 2 mM lysine in the external medium were 0.545 ± 0.020 (lysine exit) and 0.040 ± 0.003 (leucine exit). Erythrocytes were obtained from one individual representative of the HT phenotype. The external medium contained Na^+ as the principal cation.

unlabelled amino acids, is shown in Figs. 3 (LT) and 4 (HT). It can be appreciated that, whereas L-lysine exit is simulated by the presence of amino acids in the external medium (Fig. 3*A* and Fig. 4*A*), the exit of leucine is not (Fig. 3*B* and Fig. 4*B*). Average exit rates obtained from several experiments are shown in Fig. 5. These results indicate that leucine transport across system y^+L is a highly asymmetric process, the entry being favored over the exit.

The data presented in Fig. 6 compare the rates of lysine and leucine measured simultaneously in cells from 6 different individuals. It can be appreciated that, whereas L-lysine entry fluxes differ greatly, L-leucine entry fluxes are of similar magnitude (10.3 ± 1.0 nmol/L cell /min (HT) and 8.2 ± 1.4 nmol/ L cell/mm (LT) and comparable to the flux of L-lysine in LT erythrocytes. Average values for system y^+L leucine transport in this study (Na^+ medium) were (nmol/ L cell /min): 8.9 ± 0.9 (HT, $n = 7$) and 7.1 ± 0.6 (LT, $n = 13$). This observation, which at a first glance may appear in conflict with the conclusion that leucine enters through the same

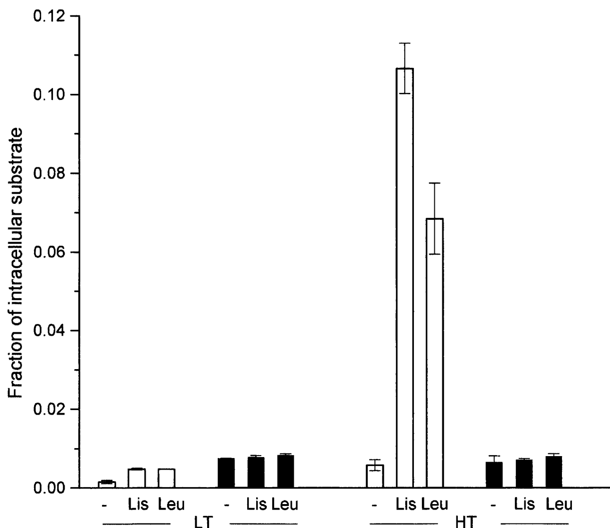

Fig. 5. Comparison of the rates of exit of L-[^{14}C]leucine (*black bars*) and L-[^{14}C]lysine (*white bars*) in LT and HT erythrocytes. Rates were measured in the absence or presence of lysine (2 mM) and leucine (10 mM) in the external medium. The intracellular concentration of lysine was 5.1 μM for HT erythrocytes (n = 4) and 4.1 μM for LT erythrocytes (n = 2) and the intracellular concentration of leucine was 6.5 μM in HT erythrocytes (n = 4) and LT erythrocytes (n = 2). Error bars represent either SD (HT) or range (LT).

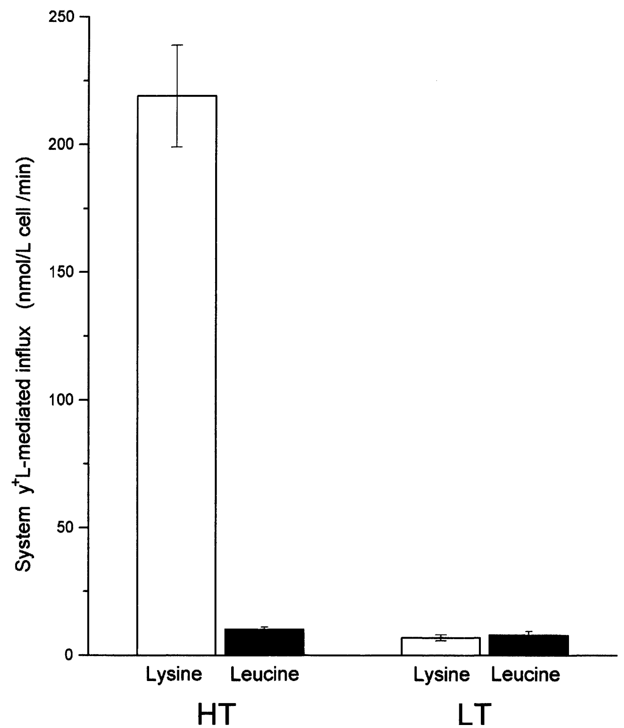

Fig. 6. Comparison of L-lysine and L-leucine y$^+$L-mediated entry rates in LT and HT erythrocytes. Values are average rates of mediated lysine influx (*white bars*) and lysine-inhibitable leucine influx (*black bars*) in HT (n = 7) and LT (n = 8) erythrocytes. The initial extracellular concentration of L-lysine and L-leucine was 1 μM and the medium contained Na$^+$ as the principal cation.

transporter as lysine, is shown in the Discussion to be consistent with the kinetic parameters that define the interactions of lysine and leucine with system y$^+$L in HT and LT erythrocytes. Interestingly, the two phenotypes could be clearly discriminated using L-leucine as a substrate in the presence of Li$^+$ (Fig. 7). As will be discussed below, this is explained by the higher affinity of L-leucine for the HT transporter in the presence of Li$^+$, than in Na$^+$ (Fig. 8) (Vargas & Devés, 2001). The V_{max} for the HT and LT erythrocytes in the presence of Li$^+$ can be estimated from the results shown in Figs. 7 and 8. The V_{max} in the presence of lithium in HT cells (1.27 μ moles/L cell/min) exceeds by 3.2 fold the rate in LT cells (0.4 μmoles/L cell/min).

Discussion

Lerner et al. (1978) and Somes et al. (1981) demonstrated that there are important individual differences in the ability of chicken to transport leucine and lysine across the erythrocyte membrane and that these differences can be emphasized by genetic selection. Surprisingly, it was found that the increase in the ratio of the magnitude of amino-acid influx in the high- and low-transport groups, observed as a result of selective breeding, varied depending on the amino acid used. Thus, the ratio of amino-acid influx in the high- and low-transport groups, in the first generation, was 4.6 for L-lysine and only 1.6 for L-leucine. In the second generation, the ratio for L-leucine transport reached 4.9 (Somes et al., 1981).

Since it has recently been proposed, on the basis of studies of L-[^{14}C]lysine transport, that system y$^+$L accounts for the high (HT) and low (LT) amino-acid transport phenotypes (Vargas & Devés, 2001), the different behavior of these two substrates of system y$^+$L must be explained. There are two simple hypothesis: 1) system y$^+$L does not carry a major proportion of the total leucine flux in these cells and 2) there are differences in the interaction of these two amino acids with system y$^+$L in HT and LT erythrocytes.

The characterization of L-leucine transport shows that a high proportion (approximately 70%) of L-leucine influx occurs through system y$^+$L (broad spectrum—Na$^+$-dependent), although another minor component, which is Na$^+$-independent and not inhibited by lysine, also is present; the minor component is similar in magnitude in the HT and LT groups.

However, the efflux of L-leucine does not show the characteristics of a system y$^+$L-mediated transport process. One of the distinctive features of system y$^+$L and other heteromeric transporters is that they

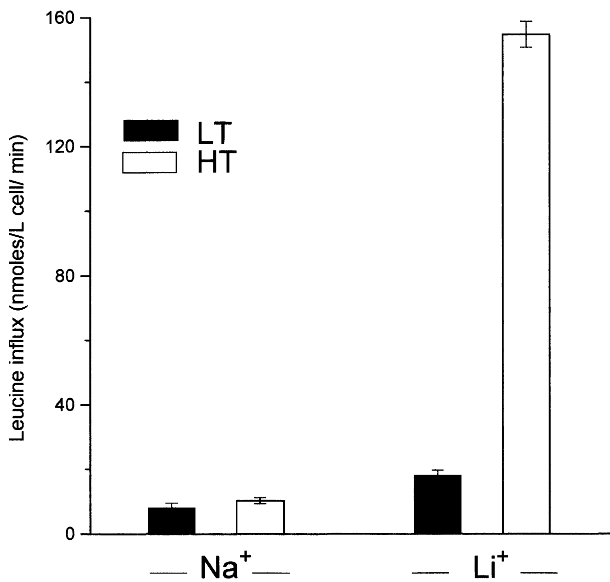

Fig. 7. Comparison of L-leucine entry rates measured in the presence of Na^+ or Li^+ in the external medium in LT (*black bars*) and HT (*white bars*) erythrocytes. The initial concentration of L-leucine was 1 μM. Average rates in the presence of Li^+ were calculated from results obtained in six independent experiments. The data for leucine uptake in the presence of Na^+ are the same as those plotted in Fig. 6.

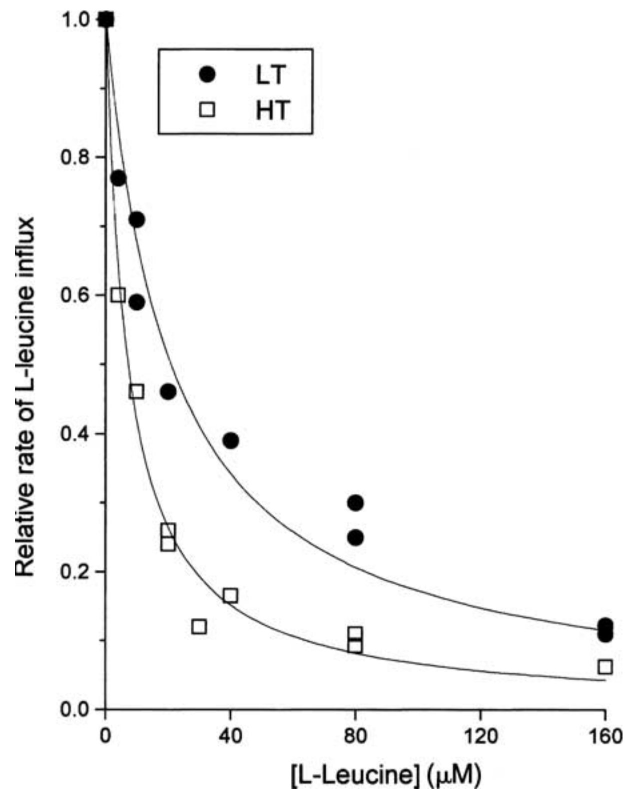

Fig. 8. Effect of unlabelled L-leucine on the relative rates of entry of L-[^{14}C]-leucine in LT (*circles*) and HT (*squares*) erythrocytes in the presence of Li^+. The concentration of L-[^{14}C]leucine was 1 μM. The calculated inhibition constants were : 20.8 ± 2.1 μM (LT, n = 2) and 7.2 ± 0.5 μM (HT, n = 3).

function as tightly coupled exchangers (Chillarón et al., 1996) and leucine exit did not exhibit the phenomenon of trans-acceleration (in HT or LT erythrocytes). Thus, leucine transport through system y$^+$L in chicken erythrocytes is highly asymmetric, the entry being favored over exit. Lack of trans-stimulation of leucine exit was also observed earlier by Chillarón et al. (1996) in oocytes whose y$^+$L activity had been enhanced by injecting the y$^+$L transporter heavy chain (4F2hc). Asymmetry is consistent with the Na^+ dependence of leucine binding; the lower concentration of Na^+ in the intracellular medium would reduce the probability of leucine binding to the inward conformation of the y$^+$L transporter. This observation is in agreement with the proposed physiological role for system y$^+$L as a mediator of the exchange of intracellular L-lysine for extracellular neutral amino acids (Bröer et al., 2000; Palacín, Borsani & Sebastio, 2001; Bauch et al., 2003).

The finding that the influx of leucine does occur largely through system y$^+$L, both in HT and LT erythrocytes, rules out the possibility that the dissimilarity in the response of leucine and lysine fluxes in selective breeding experiments is due to a differential contribution of system y$^+$L to the total flux of these amino acids.

The analysis of the effect of unlabelled L-leucine on the rates of L-[^{14}C]lysine influx and efflux suggested that the second alternative is correct, i.e., that the disparity is caused by differences in the interaction of L-lysine and L-leucine with system y$^+$L (Vargas & Devés, 2001) in the two phenotypes. It was reported earlier that, whereas in the case of lysine the affinity is greater in the HT group, the opposite occurs in the case of L-leucine ($K_{iLIS}(HT)/K_{iLIS}(LT)$ = 0.32 and $K_{iLEU}(HT)/K_{iLEU}(LT)$= 10.2). Thus, in the case of leucine, the lower relative affinity in the HT group would counterbalance the higher maximum velocity, at sub-saturating concentrations. Since the ratio of maximum velocities for both amino acids ($V_{max\ HT}/V_{max\ LT}$) has been estimated to be approximately 10 (Vargas & Devés, 2001), it can be predicted that at very low leucine concentration the rate of influx (which is proportional to V_{max}/K_m) should not differ in the two groups.

The experimental results obtained here have been found to be consistent with this prediction. Whereas the average entry rates of lysine in the HT and LT erythrocytes differed by a factor of 32, the rates of entry of L-leucine were not significantly different.

It has also been found that the ability of L-leucine to discriminate between the two groups depends on the conditions used in the assay. Thus, if leucine influx is measured in the presence of Li^+ instead of

Na^+, the rate in the HT individuals exceeds by approximately 9-fold the rate in LT individuals. This is because in the presence of Li^+ the affinity of the transporter for leucine increases and the higher capacity of HT erythrocytes to transport amino acids though system y^+L can be expressed. Therefore, the apparently complex behavior can be explained, if the differences in the interactions of lysine and leucine with the transporter are taken into consideration.

The results reported in this study confirm the finding that system y^+L accounts for high and low amino-acid transport phenotypes in chicken erythrocytes, and explain the differences found in earlier reports in the magnitude of the change induced by selective breeding in leucine and lysine transport.

No differences in terms of growth rate, size, disease resistance have been observed in animals with HT or LT phenotypes and, therefore, whether the two different phenotypes also affect other cells and tissues is not known.

The study of the molecular bases of this phenotypic variation should give, in the future, valuable information regarding the physiological role of the different molecular variants of the y^+L transporters. As is the case for other members of the family of heteromeric amino-acid transporters, the minimum functional unit of the y^+L transporters is a heterodimer composed of a heavy (4F2 hc) and a light chain (y^+LAT-1 or y^+LAT-2) (reviewed in Verrey et al., 2004).

Therefore, in theory, the HT and LT phenotypes could result from: a) differences in the expression or amino-acid sequence of the heterodimer heavy chain, b) differences in the expression or amino-acid sequence of the heterodimer light chains or c) differential involvement of an unknown modulator. The first alternative is unlikely, because the heavy subunit of the heteromeric transporters, although important for trafficking of the dimer to the membrane, does not seem to be essential for transport function per se (Reig et al., 2004). In addition, the 4F2hc heavy chain is shared by at least 6 other light subunits with different specificities and therefore mutations at this level would be expected to produce more drastic physiological consequences. The second proposal, that the HT and LT phenotypes result from modifications at the level of the light subunit, is plausible. The light subunit has been shown to be responsible for transport and also is the molecular structure that determines specificity; thus, mutations at this level could explain the observed effects. Two light subunits sustaining y^+L activity (y^+LAT-1 and y^+LAT-2) have been described in human and rodents and the two isoforms share high identity at both nucleotide and amino-acid levels (Torrents et al., 1998; Pfeiffer et al., 1999; Broer et al., 2000; Kanai et al., 2000). The y^+LAT-1 transporter is expressed preferentially in intestine and kidney and the y^+LAT-2 protein is more ubiquitous. Although the lack of specific antibodies has not allowed the identification of the molecular species responsible for system y^+L in erythrocytes, it appears to be distinct from that present in epithelia because transport through system y^+L has been found to be normal in the erythrocytes of patients presenting lysinuric protein intolerance (LPI) (Boyd et al., 2000), a genetic defect that results from mutations in the gene that encodes y^+LAT-1 (Borsani et al., 1999; Torrents et al., 1999). It has been proposed that the apparently normal transport function in the cells from these patients is most likely due to a predominant or exclusive expression of y^+LAT-2 in these cells (Sperandeo et al., 2005).

On the basis of this evidence, it would be reasonable to speculate that the HT and LT phenotypes result from mutations at the level of the light chain of the erythrocyte y^+L transporter and more specifically at the level of the y^+LAT-2 protein. There is no evidence at present to support the participation of a transport modulator. One relevant piece of information in this respect is the finding that the difference in phenotype is intrinsic to the membrane, because it persists in membrane ghosts after washing out the intracellular contents (unpublished observations). It is expected that the further investigation of the molecular bases of the HT and LT phenotype should shed light on the structure-function relationship of the y^+L transporters and its relation to pathophysiology.

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