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N-ETHYLMALEIMIDE DISCRIMINATES BETWEEN TWO LYSINE TRANSPORT SYSTEMS IN HUMAN ERYTHROCYTES

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SUMMARY

1. The sulfhydryl reagent *N*-ethylmaleimide (NEM) was shown to inactivate the low affinity lysine transporter in human erythrocytes (system y^+) without affecting the high affinity transporter (system y^+L).

2. Pre-treatment of the cells with NEM reduced the rate of entry of L-[^{14}C]lysine (1 μM) by approximately 50% (maximum effect).

3. NEM (0·2 mM) inhibited the NEM-sensitive component of the flux with mono-exponential kinetics. The inactivation rate constant (k, ±S.E.M.) was $0{\cdot}53 \pm 0{\cdot}027$ min^{-1} (25 °C). The substrate did not protect against inactivation.

4. Lysine self-inhibition experiments revealed two transport systems in untreated cells (half-saturation constants K_m; ±S.E.M.), $12{\cdot}0 \pm 1{\cdot}7$ μM and $109 \pm 15{\cdot}6$ μM) and only one high affinity system in NEM-treated cells (K_m $9{\cdot}5 \pm 0{\cdot}67$ μM), indicating that NEM inactivates system y^+.

5. The NEM-insensitive L-[^{14}C]lysine influx (system y^+L) was inhibited with high affinity by unlabelled neutral amino acids. The inhibition constant for L-leucine in sodium medium (K_i ±S.E.M.) was $10{\cdot}7 \pm 0{\cdot}72$ μM (37 °C). The system was also strongly inhibited by L-methionine, L-glutamine and with less affinity by L-phenylalanine and L-serine. *N*-methyl-L-leucine, L-proline and 2-amino-2-norbornane-carboxylic acid, a bicyclic analogue of leucine, did not exert a significant effect.

6. Lysine transport through system y^+L occurred at the same rate in Na^+, K^+ or Li^+ medium and the binding of lysine to the transporter was unaffected by Na^+ replacement.

7. The interaction of system y^+L with neutral amino acids was dependent on the cation present in the medium. The inhibition constant for leucine and glutamine increased approximately 90- and 60-fold respectively when Na^+ was replaced by K^+. Li^+ was shown to be a very good substitute for Na^+.

INTRODUCTION

Studies on the membrane transport of cationic amino acids have led to the notion that in a wide variety of tissues these molecules cross the cell membrane largely by a system known as y^+. L-Arginine, L-lysine and L-ornithine are the typical substrates of system y^+ (half-saturation constant (K_m) 0·025–0·2 mM), but it also appears to

interact with neutral amino acids with considerably lower affinity (White, 1985). This transporter was assumed to account for all the cationic amino acid transport in Ehrlich cells (Thomas, Shao & Christensen, 1971), fibroblasts (White, Gazzola & Christensen, 1982), reticulocytes (Christensen & Antonioli, 1969) and erythrocytes (Harvey & Ellory, 1989).

The idea of a single transporter serving for cationic amino acids in human erythrocytes has recently been challenged on the basis of a kinetic study of the effect of neutral amino acids on L-[^{14}C]-lysine influx (Devés, Chávez & Boyd, 1992). Neutral amino acids were found to partially inhibit lysine entry, the effect reaching a maximum at approximately 50% of the original flux (when lysine in the external medium was 1 μM). Leucine, methionine and phenylalanine were shown to be more powerful inhibitors than alanine and serine. It was proposed that lysine crosses the red cell membrane through two independent transport routes: a high affinity–low capacity transporter which recognizes both cationic and neutral amino acids (system y^+L) and a low affinity–high capacity transporter which shows similar characteristics to the previously described system y^+ (Young, Jones & Ellory, 1980).

Several recent observations in other tissues indicate that a heterogeneous population of cationic amino acid transport systems may be a frequent situation. Three separate saturable pathways for lysine have been identified in mouse blastocysts, two of which also transport neutral amino acids such as leucine, methionine and phenylalanine (Van Winkle, Campione & Gorman, 1988). These systems, which have been termed $B^{o,+}$ and $b^{o,+}$, differ regarding the cation dependence and the specificity for neutral amino acids. System $B^{o,+}$ (Van Winkle, Christensen & Campione, 1985) is sodium dependent and accepts branched species such as the leucine analogue 3-amino-endo-bicyclo[3,2,1]octane-3-carboxylic acid and relatively small amino acids such as valine. System $b^{o,+}$ (Van Winkle *et al.* 1988) is sodium independent and has a more limited substrate tolerance. This situation also appears to be the case in the placental syncytiotrophoblast where two transport systems for lysine and arginine, differing in their interaction with neutral amino acids have been described (Furesz, Moe & Smith, 1991).

It is also interesting to note that the mouse ecotropic retrovirus receptor, recently expressed in oocytes, was shown to transport cationic amino acids in a way that resembled system y^+, but was not inhibited by neutral amino acids (Kim, Closs, Albritton & Cunningham, 1991). Thus, additional systems such as those described in blastocysts, placental trophoblasts and erythrocytes appear to be required to account for the more complex interactions seen in intact membranes.

The question of whether a single pathway or multiple pathways participate in the transport of a given substrate can be conveniently addressed with the aid of specific inhibitors. By inhibitor we mean either a non-transported ligand that binds reversibly or a protein reagent that modifies functional groups. The lack of selective inhibitors has been one of the major obstacles in the description of amino acid transport. We have dealt with this problem here.

We report the functional isolation of system y^+L of human erythrocytes using *N*-ethylmaleimide (NEM). This sulfhydryl reagent selectively inactivated system y^+ leaving system y^+L functionally intact. The kinetics of the two transporters were re-examined using this new experimental tool and the results were consistent with the

conclusions reached in the previous analysis of intact cells. In addition, the specificity and cation dependence of system y^+L was investigated in more detail. As previously suggested, this system presents unusual properties in that it recognizes both cationic and neutral amino acids with very high affinity. The interaction of the transporter with neutral amino acids (but not the cationic analogues) was highly dependent on the cation present in the medium. Na^+ and Li^+ facilitated the binding; the apparent affinity observed in the presence of these ions exceeded by approximately two orders of magnitude the affinity measured in K^+.

METHODS

Experimental

Chemicals

Uniformly labelled L-[^{14}C]lysine was purchased from Amersham (approximately $1{\cdot}1 \times 10^{10}$ Bq $mmol^{-1}$), unlabelled amino acids and NEM from Sigma, and dibutylphthalate from Merck. All other chemicals were of commercial reagent grade.

Preparation of cells

Human blood was obtained fresh from donors, using heparin as an anticoagulant. The cells were spun and the plasma, buffy coat and upper layer of cells removed by aspiration. The red cells were washed four times with 5 mM sodium phosphate buffer (pH 6·8) containing 150 mM NaCl and 4 mM KCl and incubated to eliminate endogenous amino acids (2·5% haematocrit, 15 h at 25 °C). This buffer was used in all experiments unless otherwise indicated. In long incubations 0·02% chloramphenicol was included.

Treatment with NEM

Washed erythrocytes (haematocrit 2·5%) were incubated for varying times with NEM at 25 °C. The reaction was terminated by addition of 2-mercaptoethanol (10 mM final concentration). The cells were then washed, packed and assayed for transport. In one experiment, lysine (1 mM) was added to the buffer, the cells were incubated (37 °C) for a period of 3·5 h to allow for lysine equilibration and then treated with NEM as explained above.

Measurement of entry rates

Entry was followed by adding packed cells (0·3 ml) at time zero to a solution of L-[^{14}C]lysine in isotonic saline (10% haematocrit, 37 °C). Three samples of the suspension (0·8 ml) were withdrawn at intervals (up to 4–5 min) and placed in tubes containing dibutylphthalate (0·5 ml). After centrifugation (45 s, 11000 *g*) the cells sedimented below the organic layer. The aqueous layer was taken off by aspiration and the walls of the tubes were thoroughly washed to eliminate contaminating radioactivity. Dibutylphthalate was removed and the cells were precipitated by addition of 5% trichloroacetic acid (0·6 ml). The suspension was centrifuged, and the radioactivity in the supernatant (0·5 ml) determined by scintillation counting.

All determinations were performed in duplicate runs and rates were estimated from linear regression analysis of six time points. The uptake progress curves show a positive intercept. This is due to a small amount of original extracellular medium which surrounds the cells in the pellet (approximately 4·5% of total pellet volume). The washing procedure does not remove this layer of fluid which is below the organic layer. The intercept therefore represents extracellular radioactivity associated with the cells at all times. The common intercept obtained in the presence or absence of transport inhibitors (see Fig. 2) shows that this is the case. Estimations of this quantity at time zero agree with this interpretation.

Theory

Transport rate equation for two systems acting in parallel

The rate (v) for the transport of a substrate (S) through two transporters, y^+ and y^+L, is expressed as the sum of two Michaelis–Menten-type functions. The corresponding kinetic parameters are identified by subscripts Y and YL.

$$v = v_Y + v_{YL}. \tag{1}$$

$$v = \frac{V_{maxY}[S]}{K_{mY}+[S]} + \frac{V_{maxYL}[S]}{K_{mYL}+[S]}. \tag{2}$$

V_{max} represents the maximum rate of transport and K_m the half-saturation constant.

Irreversible inhibition

If system y^+ is assumed to be fully and selectively inactivated by an irreversible inhibitor, the relative transport rate, in the presence of the inhibitor (v_I) and in its absence (v_o), can be written as:

$$\frac{v_I}{v_o} = \frac{V_{maxYL}/(K_{mYL}+[S])}{V_{maxY}/(K_{mY}+[S]) + V_{maxYL}/(K_{mYL}+[S])}. \tag{3}$$

Reversible inhibition

The self-inhibition of L-[^{14}C]lysine influx (1 μM) by unlabelled lysine ([I]) in untreated cells was analysed according to the following equation (two system model) (Devés *et al.* 1992):

$$\frac{v_I}{v_o} = \frac{1/(1+[I]/K_{mYL}) + F/(1+[I]/K_{mY})}{1+F}, \tag{4}$$

where

$$F \text{ (the permeability ratio)} = \frac{V_{maxY} K_{mYL}}{K_{mY} V_{maxYL}}. \tag{5}$$

The reversible inhibition of L-[^{14}C]lysine influx (1 μM) by unlabelled amino acids in NEM-treated cells was analysed with the expression given below (one system model):

$$\frac{v_I}{v_o} = \frac{1}{1+[I]/K_{iYL}}. \tag{6}$$

RESULTS

Human erythrocytes were preincubated with 1 mM NEM for 1, 2·8 and 5 min and then tested for L-lysine influx (1 μM). The flux was reduced by approximately 50% with all incubation protocols, suggesting that the lysine transporters in erythrocytes are heterogeneous with respect to NEM sensitivity. The measured rates (μmol (l cells)$^{-1}$ min^{-1} ± S.E.M.) were: for the control, 0·060 ± 0·0002; NEM 1 min, 0·032 ± 0·001; NEM 2·8 min, 0·033 ± 0·0004 and NEM 5 min, 0·036 ± 0·0003. At this concentration, therefore, the maximum inhibition was attained in less than 1 min.

The kinetics of the NEM reaction were studied using a lower concentration of the reagent. In Fig. 1 the effect of 0·2 mM NEM on the rate of L-lysine entry is plotted against the time of treatment with the sulfhydryl reagent. The inactivation curve is clearly biphasic; the rate is seen to decline until it reaches a constant level at about 40% of the original flux. If the non-inhibitable component is subtracted (inset), the inactivation kinetics become mono-exponential. The inactivation rate constant (k) under these conditions (± S.E.M.) was 0·53 ± 0·027 min^{-1}. With 0·1 mM NEM the rate constant was 0·23 ± 0·019 min^{-1}, ($n = 2$). The rate of inactivation was also measured in the presence of 1 mM lysine (inside and outside the cell). The substrate was not found to protect against NEM (k, 0·55 ± 0·022 min^{-1}, $n = 1$).

As mentioned in the Introduction, two transport systems for lysine have recently been described in human erythrocytes on the basis of their differential interactions

with neutral amino acids (Devés *et al.* 1992). In order to find out whether or not the partial inhibition caused by NEM is related to any of these two systems, the effect of simultaneous and separate additions of leucine and NEM was compared (Fig. 2). The inhibitions caused by L-leucine and NEM were found to be additive suggesting

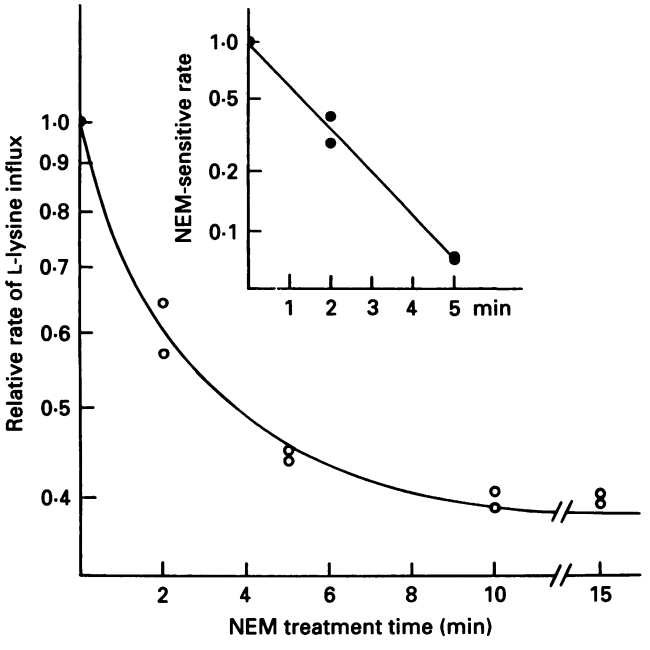

Fig. 1. Relative rates of L-[^{14}C]lysine influx in cells treated with NEM for varying times at 25 °C. The inset shows a replot of these results after subtracting the NEM-resistant flux (value after 15 min of incubation). The lysine concentration used to estimate the entry rate was 1 μM. Results for two cell samples are shown and the data points represent the pooled separate results from the different samples. All uptake rates were determined in duplicate runs at 37 °C as explained in the Methods. Control uptake rates (μmol (l cells)$^{-1}$ min^{-1} $\pm$ S.E.M.) were 0·070 $\pm$ 0·0025 and 0·075 $\pm$ 0·0019. Inactivation rate constants (k) were calculated from the following equation: $\ln v/v_0 = -kt$, where v/v_0 is the relative transport rate in the presence and absence of NEM.

that NEM inactivates the leucine-insensitive component of the flux or system y^+, but not the leucine-sensitive component or system y^+L. A small difference is seen for the leucine-sensitive flux measured in the absence or presence of NEM (0·032 and 0·028 μmol (l cells)$^{-1}$ min^{-1} respectively). This could be taken to mean that NEM inhibits the leucine-sensitive flux by 12·5%. The difference, however, arises from the fact that leucine interacts very weakly with system y^+ (K_i, 30·4 $\pm$ 7·9 mM) (Devés *et al.* 1992). At a leucine concentration of 4 mM, system y^+ is expected to be inhibited by 11·5%. The leucine-inhibited flux corresponds to system y^+L plus a small percentage of the flux through system y^+. The corrected rates are (μmol (l cells)$^{-1}$ min^{-1}): total, 0·064; system y^+, 0·036; system y^+L, 0·028.

The hypothesis that NEM is a selective inhibitor for system y^+ can be tested

further by examining the interaction of the transporter with lysine in treated and untreated cells. Since system y^+ has been shown to bind lysine with lower affinity than system y^+L, the lysine self-inhibition profiles should change after NEM treatment. The results, shown in Fig. 3, agree with this prediction. Whereas two

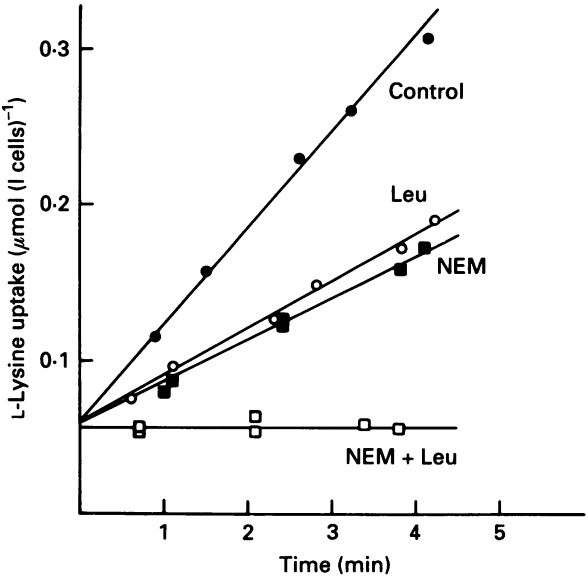

Fig. 2. Cells treated with 0·2 mM NEM for 10 min at 25 °C and untreated cells were assayed for transport in the presence or absence of L-leucine (Leu, 4 mM). The lysine concentration in the external medium was 1 μM and the temperature 37 °C. The rates calculated from the slopes of the lines are (μmol (l cells)$^{-1}$ min^{-1} ± S.E.M.): control, 0·064 ± 0·001; leucine, 0·032 ± 0·0005; NEM, 0·028 ± 0·0005.

transport systems can be distinguished in control cells (with half-saturation constants (± S.E.M.) of K_{mY}, 109 ± 15·6 μM and K_{mYL}, 12·0 ± 1·7 μM), only one high affinity system is apparent after NEM reaction (K_{mYL}, 9·5 ± 0·67 μM). As predicted, the lower affinity component of control cells is eliminated by NEM treatment, showing that the protein reagent is acting on system y^+, the lower affinity route for lysine entry. The lysine self-inhibition profile for NEM-treated cells with K^+ replacing Na^+ in the external medium is also shown. The calculated half-saturation constant is 8·65 ± 0·33 μM, a similar value to that obtained in the presence of sodium.

The conclusion that NEM selectively inactivates a low affinity transporter is also supported by the results shown in Table 1. In this experiment cells were treated with NEM (0·2 mM, 10 min) and then split into three fractions to be assayed for lysine influx with 1, 20 and 100 μM lysine in the external medium. NEM was found to inhibit a larger proportion of the flux at higher lysine concentrations. This is expected, because as the substrate concentration is raised the fraction of the flux carried by system y^+ should increase. As explained in the Discussion, this behaviour is in quantitative agreement with the kinetic parameters estimated for the two systems.

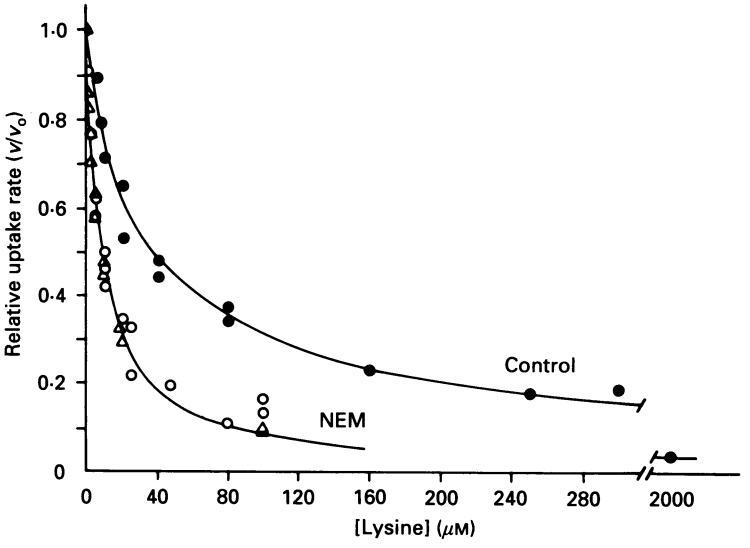

Fig. 3. Relative rates of L-[^{14}C]lysine entry in the presence of varying concentrations of unlabelled L-lysine in the external medium in control (●) and NEM-treated cells (0·2 mM, 10 min) with Na^+ (○) or K^+ (△) in the external medium. Labelled lysine concentration was kept fixed at 1 μM. All rates were calculated from six data points and the data points represent the pooled separate results from different samples. Entry rates in the absence of unlabelled lysine (μmol (l cells)$^{-1}$ min^{-1} ± S.E.M.) were: control + Na^+ ($n = 3$), 0·052 ± 0·0016, 0·054 ± 0·0004, 0·068 ± 0·005; NEM + Na^+ ($n = 3$), 0·025 ± 0·0012, 0·028 ± 0·001, 0·043 ± 0·0022; NEM + K^+ ($n = 2$), 0·022 ± 0·001, 0·029 ± 0·0015. Some of the measurements in control cells ($n = 1$) were shown in a previous publication (Devés *et al.* 1992). Half-saturation constants were calculated by non-linear regression analysis of the data, according to eqn (4), with $F = 1$ (control) or eqn (6) (treated cells) as explained in the Methods. The external medium contained either 154 mM NaCl, 5 mM sodium phosphate or 154 mM KCl, 5 mM potassium phosphate (pH 6·8, 37 °C) as indicated.

TABLE 1. Kinetic parameters for systems y^+ and y^+L calculated from NEM effects

Cell	[Lysine] (μM)	Relative rates after NEM treatment (v_I/v_o): Experimental (± S.E.M.)	Theoretical	$\frac{V_{maxY}/K_{mY}}{V_{maxYL}/K_{mYL}}$	$\frac{V_{maxY}}{V_{maxYL}}$
1	1	0·64 ± 0·03	—	0·56	5·08
	20	0·45 ± 0·04	0·44		
	100	0·33 ± 0·02	0·29		
2	1	0·39 ± 0·03	—	1·56	14·16
	20	0·18 ± 0·02	0·22		
	100	0·13 ± 0·03	0·12		

Relative lysine entry rates into untreated and treated cells (0·2 mM NEM, 10 min) were measured at 1, 20 and 100 μM lysine in the external medium. The table lists the relative rates after NEM treatment as a function of lysine concentration in two different cell samples. The 'permeability ratio' $((V_{maxY}/K_{mY})/(V_{maxYL}/K_{mYL}))$ was calculated from the v_I/v_o value at 1 μM lysine on the basis of eqn (7) (Discussion). The ratio of maximum rates (V_{maxY}/V_{maxYL}) was estimated from the 'permeability ratio' and the half-saturation constants measured in Fig. 3 (K_{mY}, 109 and K_{mYL}, 12). Theoretical values for the relative inactivation rates, estimated with these parameters and eqn (3), are compared with experimental observations.

In subsequent experiments system y^+L was characterized, in NEM-treated cells, with respect to its interaction with neutral amino acids and monovalent cations. The effect of varying concentrations of L-leucine on the lysine entry rate is shown in Fig. 4. The ability of the transporter to recognize leucine is seen to be dependent on the

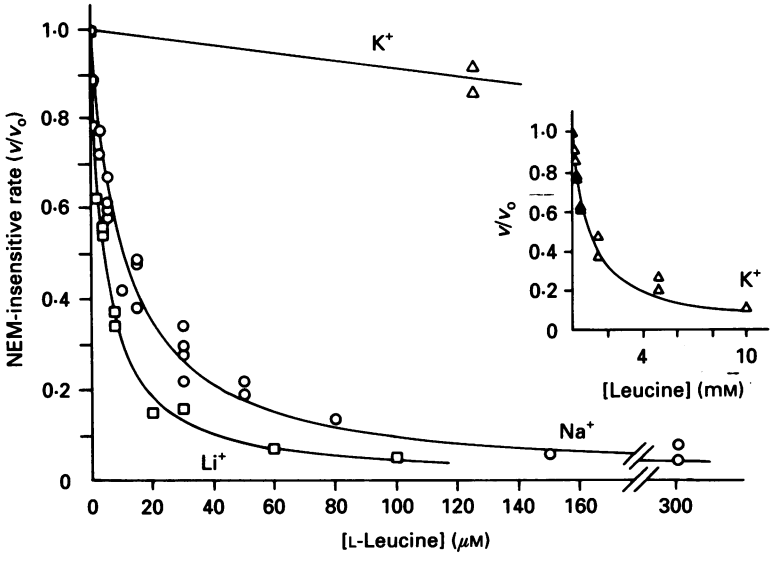

Fig. 4. Relative rates of L-[^{14}C]lysine entry into NEM-treated cells in the presence of varying concentrations of unlabelled L-leucine in the external medium. The cells were treated with 0·2 mM NEM for 10 min (25 °C). The L-[^{14}C]lysine concentration was 1 μM and the temperature 37 °C. The experiment was performed with Na^+ (154 mM NaCl, 5 mM sodium phosphate), K^+ (154 mM KCl, 5 mM potassium phosphate) or Li^+ (154 mM LiCl, 5 mM potassium phosphate) in the external medium. All rates were calculated from six time points and the data represent the pooled separate results from different samples. Average values for entry rates in the absence of unlabelled leucine (μmol (l cells)$^{-1}$ min^{-1} ± S.D.) were: Na^+, 0·041 ± 0·0045 (n = 3); K^+, 0·032 ± 0·0042 (n = 2); Li^+, 0·027 (n = 2). Inhibition constants (K_i) were calculated by non-linear regression analysis of the data, according to eqn (6).

monovalent cation present in the medium as was previously found with intact cells (Devés *et al.* 1992). In Na^+ medium the transporter recognizes leucine with very high affinity, the inhibition constant (K_i ± S.E.M.) was 10·7 ± 0·7 μM. In K^+ medium, however, the affinity decreases dramatically and much higher concentrations are required to produce an effect (K_i, 983·0 ± 80·7 μM). Interestingly, Li^+ was shown to be a good substitute for Na^+; the apparent affinity measured with this cation was 2·4-fold higher than the apparent affinity measured in Na^+ (K_i, 4·52 ± 0·26 μM).

The effect of another neutral amino acid, L-glutamine, on system y^+L, in the presence of different monovalent cations in the medium, is shown in Fig. 5. As found for leucine, the binding of glutamine was very much reduced when Na^+ was replaced by K^+, and Li^+ was also a good substitute ion. The inhibition constants calculated from this experiment were: 29·3 μM (Na^+), 33·0 μM (Li^+) and 1·8 mM (K^+). The results presented in Fig. 6 show that the lysine entry rate is virtually the same with Na^+,

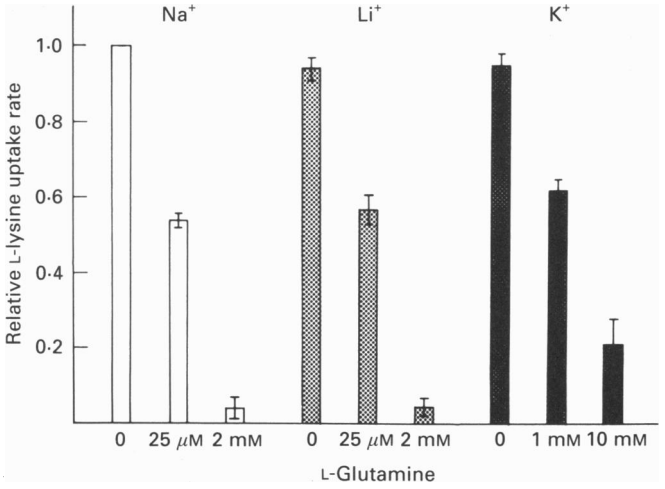

Fig. 5. Effects of unlabelled L-glutamine on the rate of entry of L-[^{14}C]lysine, into NEM-treated cells, with either Na^+, K^+ or Li^+ in the external medium. Cells were treated with 0·2 mM NEM for 10 min (25 °C). Lysine concentration was 1 μM and the temperature 37 °C. Rates are expressed in relative units with respect to the control rate in the presence of Na^+. The data represent the average value (±S.D.) for two cell samples. The control rates (μmol (l cells)$^{-1}$ min^{-1} ± S.E.M.) in Na^+ medium were: 0·04 ± 0·001 and 0·03 ± 0·0007. The external medium contained either 154 mM NaCl, 5 mM sodium phosphate; 154 mM KCl, 5 mM potassium phosphate or 154 mM LiCl, 5 mM lithium phosphate (pH 6·8) as indicated.

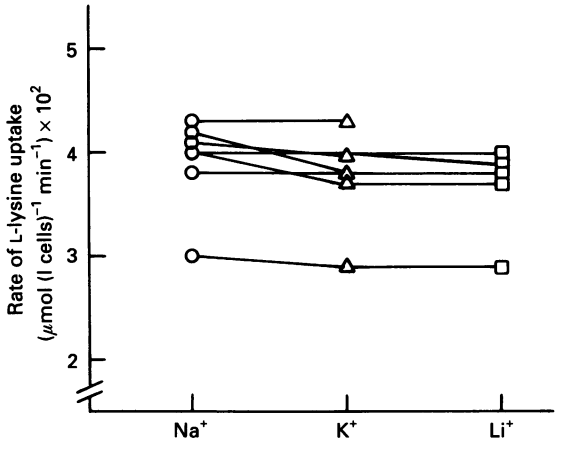

Fig. 6. Rates of lysine entry (1 μM, 37 °C) in cells treated with 0·2 mM NEM for 10 min (25 °C) with Na^+, K^+ or Li^+ in the external medium. Rates were determined from six time points in seven different cell samples. The error (S.E.M.) of each determination was typically around 4–6%. The external medium contained either 154 mM NaCl, 5 mM sodium phosphate; 154 mM KCl, 5 mM potassium phosphate or 154 mM LiCl, 5 mM lithium phosphate (pH 6·8) as indicated.

K^+ or Li^+ in the external medium. Since the half-saturation constant for lysine is also unaffected by sodium removal, it can be concluded that the translocation and binding of lysine occur equally well in Na^+, Li^+ or K^+.

The specificity of system y^+L for other neutral amino acids and D-isomers can be deduced from the results presented in Table 2. Inhibition constants were calculated from the relative transport rates in the presence and absence of competing amino acid on the basis of eqn (6). The lower limits given for the inhibition constants were estimated assuming that 10% inhibition would have been experimentally observed.

TABLE 2. Inhibition of NEM-resistant lysine influx by unlabelled amino acids

Inhibitor	Concentration	Percentage inhibition (±S.D.)	K_i (mM)
L-Glutamine	15 μM	38·0±1·4	0·024
L-Glutamine	2 mM	94·5±2·1	
L-Methionine	25 μM	57·0±5·6	0·019
L-Methionine	3 mM	95·5±3·5	
L-Serine	700 μM	57·5±3·5	0·517
L-Serine	5 mM	88·5±3·5	
L-Phenylalanine	150 μM	56·0±1·4	0·118
L-Phenylalanine	5 mM	97·5±0·71	
L-Proline	500 μM	5·7±4·6	
L-Proline	2 mM	6·2±4·0	> 18
D-Leucine	5 mM	35·5±3·5	9
D-Lysine	0·5 mM	76·5±0·7	0·15
D-Lysine	5 mM	92·5±4·9	
BCH	5 mM	16·5±3·5	25
N-methyl-L-leucine	5 mM	8·5±6·4	> 45

The values for percentage inhibition are means (±S.D.) for duplicate determinations in two different samples of erythrocytes. The K_i was calculated as explained in the text. The cells were treated with 0·2 mM NEM for 10 min and the lysine concentration in the assay was 1 μM. The external medium contained 150 mM NaCl, 4 mM KCl and 5 mM sodium phosphate (pH 6·8).

DISCUSSION

Inactivation of system y^+ by NEM

The experiments presented in this paper confirm the existence of two lysine transport systems with differing substrate specificities in the human erythrocyte, an idea recently proposed to explain the partial inhibitory effect of neutral amino acids on lysine entry fluxes (Devés *et al.* 1992). NEM was shown to act as a selective inhibitor of system y^+, a low affinity–high capacity system which is highly specific for cationic amino acids. The other system which recognizes cationic and neutral amino acids with high affinity (system y^+L), was unaffected by NEM. This conclusion is supported by different observations: (1) NEM acts as a partial inhibitor of lysine influx, (2) the fraction of the flux that is inhibited by NEM is insensitive to neutral amino acids, whereas the residual flux is abolished by low concentrations of these amino acids, (3) the kinetic constants determined for systems y^+ and y^+L quantitatively explain the effects of NEM and (4) the NEM-resistant flux shows all the characteristics described for system y^+L in intact cells.

The kinetics of the inactivation by NEM are consistent with modification of a single functional group in the transport protein (Fig. 1). The reactivity of this group

(k, 0·53 min^{-1} at 0·2 mM NEM and 25 °C) is high compared to observations made in other transport proteins such as the choline carrier (Devés & Krupka, 1981) and the ASC amino acids transporter in the same cells (Al-Saleh & Wheeler, 1982). The choline carrier is inactivated by 1 mM NEM with a k of $0{\cdot}197 \pm 0{\cdot}015\ min^{-1}$ at 37 °C. A comparable value, $0{\cdot}23 \pm 0{\cdot}019\ min^{-1}$, was obtained for system y^+, at a concentration of 0·1 mM and only 25 °C. The reactivity of system ASC is even more sluggish. Treatment for 1 h at 37 °C was required to inactivate approximately half of the carrier with 1 mM NEM. Assuming a mono-exponential decay, this gives a k of $0{\cdot}01\ min^{-1}$.

The rate of inactivation was unaffected by the presence of 1 mM lysine at equilibrium and thus, it can be concluded that the reactive functional group lies outside the substrate site. This is also the case with the choline carrier (Devés & Krupka, 1981), where NEM has been proposed to react with an SH group located in the inner gated channel of the carrier which is involved in the translocation mechanism (Krupka & Devés, 1986).

Determination of the kinetic parameters of systems y^+ and y^+L using NEM

A simple experimental strategy using NEM was applied to estimate the kinetic parameters for lysine transport through systems y^+ and y^+L.

The effect of NEM at low substrate concentrations depends directly on the permeability ratio $(V_{maxY}/K_{mY})/(V_{maxYL}/K_{mYL})$ for the two systems. This can be intuitively grasped if we consider that the transport rate constant at low substrate concentrations is directly proportional to V_{max}/K_m; the permeability ratio thus determines the relative contribution of systems y^+ and y^+L to the total flux under these conditions.

This fact can be more clearly appreciated in the following equation describing the effect of NEM on lysine entry at low substrate concentrations (simplified from eqn (3) assuming that $[S] \ll K_m$) and where v_I represents the rate after NEM treatment and v_o the rate of control cells:

$$\frac{v_I}{v_o} = \frac{1}{(V_{maxY}/K_{mY})/(V_{maxYL}/K_{mYL}) + 1}. \tag{7}$$

It can be seen that the permeability ratio can be directly estimated from the relative transport rates of treated and untreated cells (measured at low substrate concentration). Permeability ratios for two cell samples are listed in Table 1. The cells used in this experiment had the highest (0·64) and the lowest (0·39) values of v_I/v_o respectively in thirteen samples analysed. The mean value (±S.D.) for the relative transport rate after NEM treatment was $0{\cdot}51 \pm 0{\cdot}086$ ($n = 13$). The average permeability ratio was therefore 0·96. This means that on average, at low substrate concentrations, the two systems make approximately equal contributions to the rate of entry.

The relative maximum velocities for the two systems can be calculated from the permeability ratios and half-saturation constants (determined in Fig. 3). As shown in Table 1, in the two cases analysed, the maximum velocity of system y^+ exceeded that of system y^+L, but the magnitude of the difference varied. With one cell sample

V_{maxY}/V_{maxYL} was 5·08, with the other, 14·16. This result probably reflects variations in the relative abundance of the two transporters in individual cell samples. The ratio of maximum velocities for thirteen individual cell samples was 9·1.

The consistency of the calculated kinetic parameters can be tested by their capacity to predict the NEM effect at higher lysine concentrations (20 and 100 μM). If NEM is inhibiting the lower affinity and higher capacity system, a larger proportion of the flux should be inhibited at higher substrate concentrations, because under these conditions more of the flux will be carried by the NEM-sensitive pathway. Theoretical values of v_I/v_0, calculated on the basis of eqn (3), are compared to the experimental values in Table 1. The observed rates are consistent with the predictions and NEM is seen to inhibit a larger proportion of the flux as the lysine concentration in the assay is raised.

Specificity of system y^+L

The NEM-resistant influx of L-[^{14}C]lysine was found to be inhibited by low concentrations of unlabelled neutral amino acids (Figs 4 and 5, and Table 2), a distinctive characteristic of system y^+L. L-Leucine, L-methionine and L-glutamine are preferred to L-phenylalanine and L-serine. The system does not tolerate amino-group substitution, as shown by the lack of effect of 5 mM *N*-methyl-L-leucine and does not interact with bicyclic compounds such as 2-amino-2-norbornane-carboxylic acid (BCH). L-Proline also failed to interact with system y^+L. It is interesting to note that the system appears to be considerably more stereospecific with respect to leucine than lysine. The apparent affinity ($1/K_i$) for L-lysine exceeds that for D-lysine (by 12·5 times). For leucine, this factor is approximately 800. The mechanistic implications of this observation should be explored in the future.

The specificity of system y^+L in general resembles that of system $b^{0,+}$ described in blastocysts by Van Winkle *et al.* (1988). System $b^{0,+}$ has been shown to transport lysine and leucine with a half-saturation constant of around 100 μM and to prefer amino acids such as leucine, tryptophan and phenylalanine rather than valine or the cyclic analogues of leucine. Proline is also excluded from system $b^{0,+}$. Recently two closely related cDNAs (79% identity in the nucleotide sequence) have been isolated from rat and rabbit kidney and shown to stimulate transport of neutral and cationic amino acids in oocytes following injection of the derived RNAs (Wells & Hediger, 1992; Bertran *et al.* 1992). Although it is not yet clear whether the cDNA encodes for a constituent element of the transporter or an activator of the system, the characteristics of the induced activity clearly resemble those of system $b^{0,+}$.

In spite of a similar substrate specificity, an important difference exists between system $b^{0,+}$ and system y^+L regarding their interactions with monovalent inorganic cations. In the case of system $b^{0,+}$ (and the homologous systems in the kidney) binding of both cationic and neutral amino acids was shown to be Na^+ independent (Van Winkle *et al.* 1988; Wells & Hediger, 1992; Bertran *et al.* 1992). In the case of system y^+L the situation is more complex and the effect of monovalent cations differs for cationic and neutral amino acids. Whereas lysine transport occurs at virtually the same rate in Na^+, K^+ or Li^+ medium (Figs 5 and 6) and the binding of lysine is unaffected by sodium replacement (Fig. 3), the association with neutral amino acids is strictly dependent on the cation present (Figs 4 and 5). The inhibition constant for leucine and glutamine increases by approximately 90- and 60-fold respectively if Na^+

is replaced by K^+. Worth noticing is the fact that Li^+ is a very good substitute for Na^+. In fact the inhibition constant for leucine is lower in Li^+ than in Na^+ medium. With glutamine, Na^+ and Li^+ are equally efficient in facilitating transport.

This behaviour is reminiscent of the early observations in Ehrlich cells where neutral amino acid inhibition of cationic amino acid influx depends on the presence of an alkali metal ion and where Na^+ and Li^+ are better than K^+ (Thomas *et al.* 1971). In these cells, however, the values of K_i at physiological concentrations of sodium are 50- to 100-fold larger than the corresponding parameters for cationic amino acids. Christensen and co-workers originally proposed that alkali metal ions serve as a substitute for the positively charged side chain of the cationic amino acids (Christensen & Antonioli, 1969) and this is most likely to be the mechanism here.

An interaction between dibasic, neutral amino acids and Na^+ binding has also been observed for the sodium-dependent ASC system of erythrocytes. System ASC binds alanine, serine, cysteine and also dibasic amino acids but with lower affinity. On the basis of inhibition studies using harmaline it was proposed that the positive charge on the dibasic amino acid side chain occupies the sodium-binding site on the transporter (Young, Manson & Fincham, 1988). Li^+ does not substitute for Na^+ in system ASC (Al-Saleh & Wheeler, 1982).

System y^+L, therefore, appears to be the only system described so far that recognizes cationic and neutral amino acids with high affinity and shows differential effects of monovalent cations in the interaction with neutral and dibasic amino acids.

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