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Cation and harmaline interactions with Na^+-independent dibasic amino acid transport system y^+ in human erythrocytes and in erythrocytes from a primitive vertebrate the pacific hagfish (*Eptatretus stouti*)

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Transport systems y^+, asc and ASC exhibit dual interactions with dibasic and neutral amino acids. For conventional Na^+-dependent neutral amino acid system ASC, side chain amino and guanido groups bind to the Na^+ site on the transporter. The topographically equivalent recognition site on related system asc binds harmaline (a Na^+-site inhibitor) with the same affinity as asc (apparent K_i range 1–4 mM), but exhibits no detectable affinity for Ha. Although also classified as Na^+-independent, dibasic amino acid transport system y^+ accepts neutral amino acids when Na^+ or another acceptable cation is also present. This latter observation implies that the y^+ translocation site binds Na^+ and suggests possible functional and structural similarities with ASC/asc. In the present series of experiments with human erythrocytes, system y^+-mediated lysine uptake (5 μM, 20°C) was found to be 3-fold higher in isotonic sucrose medium than in normal 150 mM NaCl medium. This difference was not a secondary consequence of changes in membrane potential, but resulted from Na^+ functioning as a competitive inhibitor of transport. Apparent K_m and V_{max} values for lysine transport at 20°C were 15.2 μM and 183 μmol/l cells per h, respectively, in sucrose medium and 59.4 μM and 228 μmol/l cells per h in Na^+ medium. Similar results were obtained with y^+ in erythrocytes of a primitive vertebrate, the Pacific hagfish (*Eptatretus stouti*), indicating that Na^+-inhibition is a general property of this class of amino acid transporter. At a permeant concentration of 5 μM, the IC_{50} value for Na^+-inhibition of lysine uptake by human erythrocytes was 27 mM. Other inorganic and organic cations, including K^+ and guanidinium$^+$, also inhibited transport. In parallel with its actions on ASC/asc harmaline competitively inhibited lysine uptake by human cells in sucrose medium. As predicted from mutually competitive binding to the y^+ translocation site, the presence of 150 mM Na^+ increased the harmaline inhibition constant (K_i) from 0.23 mM in sucrose medium to 0.75 mM in NaCl medium. We interpret these observations as further evidence that y^+, asc and ASC represent a family of closely related transporters with a common evolutionary origin.

Introduction

Transport of dibasic amino acids across the plasma membrane of many vertebrate cell types, including human erythrocytes, is mediated by a specific equilibrative dibasic amino acid transporter y^+ [1–3]. In contrast, the major route for dibasic amino acid permeation in horse and in sheep erythrocytes is via system asc, a novel class of Na^+-independent neutral amino acid transporters exhibiting close functional similarities to classical Na^+-dependent system ASC [2,4,5]. ASC and asc exhibit equivalent specificities for neutral amino acids of intermediate size and both possess specific recognition sites for side chain amino and guanido groups [1,4–7]. For ASC, this recognition site is the site of Na^+-binding to the transporter and can be competitively blocked by the halucinogenic alkaloid harmaline [8]. The equivalent recognition site on asc binds harmaline with the same apparent affinity as

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system ASC, but does not significantly interact with Na^+ (or other cations) as judged by the finding that the transporter functions normally in isotonic sucrose medium, both with respect to neutral and dibasic amino acid transport [8]. Critically, Na^+ was found to have no measurable effect on the apparent affinity of the transporter for L-lysine. System asc_1 variants from different equine species transport this amino acid with apparent K_m values as low as 1 mM (Young, J.D., and Fincham, D.A., unpublished data).

Although classified as Na^+-independent, system y^+ accepts small neutral amino acids when Na^+ or another acceptable cation is also present in the extracellular medium [3,9–12]. As for ASC, the combination (neutral amino acid + cation) is considered to be structurally equivalent to dibasic amino acid, implying that the y^+ translocation site exhibits a significant affinity for Na^+ and other cations. To explore this possibility, and to investigate further possible functional and structural relationships between this system and ASC/asc, we report here an investigation of the effects of Na^+, other inorganic cations and harmaline on lysine transport by human erythrocytes. Parallel studies of the effects of Na^+ on system y^+-mediated transport of lysine by erythrocytes from a primitive vertebrate, the Pacific hagfish (*Eptatretus stouti*), are also presented.

Material and Methods

Erythrocytes. Human blood samples were collected from healthy volunteers by venipuncture into heparin, and the erythrocytes prepared for transport experiments by washing three times with 20 volumes of incubation medium containing 5 mM Mops (4-morpholinepropanesulphonic acid) (titrated to pH 7.5 at 20°C with KOH), 5 mM D-glucose and either 300 mM sucrose or 150 mM NaCl. The buffy coat was discarded.

Hagfish (*Eptatretus stouti*) were trapped at 160–200 m in Trevor Channel, Barkley sound, Bamfield, B.C., Canada and shipped to the University of Alberta where they were maintained in synthetic seawater until bled from the subcutaneous sinus into heparinised tubes using MS222 (500 mg/l seawater) as anaesthetic. Hagfish erythrocytes were treated as described for human erythrocytes except that the NaCl concentration of the incubation medium was increased from 150 mM to 500 mM and mannitol (1M) replaced sucrose as NaCl substitute.

Lysine transport studies. L-[U-^{14}C]Lysine uptake (5–200 μM extracellular concentration) by human erythrocytes was measured at 20°C (30 s–30 min incubation) by a previously described ice-cold-stop washing procedure [13]. Initial uptake rates were determined in triplicate using a 2.5-min incubation period. Except for Fig. 1, all data refer to system y^+-mediated uptake, determined as that fraction of permeant uptake sensitive to inhibition by 10 mM non-radioactive L-arginine. Kinetic constants were determined by non-linear regression analysis (Enzyfitter Programme, Elsevier-Biosoft). Uptake of L-lysine by hagfish erythrocytes was measured at 10°C by an oil-stop procedure [13] using taurine as extracellular space marker [14].

Materials. L-[U-^{14}C]Lysine and [U-^{14}C]taurine were purchased from Amersham International plc, Amersham, Bucks, U.K. Non-radioactive amino acids, harmaline and valinomycin were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. and St. Louis MO, U.S.A. All other reagents were of analytical grade.

Results

Na^+-interactions with amino acid and other solute transporters are usually considered in the context of Na^+-activation and are typically investigated under experimental conditions where medium Na^+ is isosmotically replaced by other inorganic (eg. K^+) or organic (eg. choline$^+$) cations. Such cation substitutions, although appropriate in many situations of Na^+: solute cotransport, may fail to reveal general cation-inhibitory effects on transporter function. In the present study of Na^+ and harmaline interactions with human erythrocyte dibasic amino acid transport system y^+, we replaced NaCl with isosmotic sucrose, an approach previously used by us in studies of cation interactions with horse red cell system asc [8]. In the case of hagfish erythrocytes, NaCl was replaced with isosmotic mannitol. Incubation media were designed to contain a low MOPS concentration (5 mM) to minimise the amount of balancing cation required to buffer the medium pH at 7.5. With the exception of this necessary low concentration of K^+ (3.6 mM): the sucrose and mannitol media were totally cation-free. K^+ was chosen in the preference to Na^+ in anticipation that cation interactions with y^+ might be Na^+-specific [11]. An incubation temperature of 20°C was selected for the human erythrocyte experiments to facilitate determination of initial uptake rates at low permeant concentrations. To be consistent with previous amino acid transport studies [14], L-lysine uptake by hagfish erythrocytes was measured at 10°C.

Effects of Na^+ on the kinetics of lysine transport by human erythrocytes

Fig. 1 compares time-courses of L-lysine uptake (5 μM initial extracellular concentration) by human erythrocytes suspended in isotonic NaCl and sucrose media. In contrast to what would be expected for a conventional Na^+-dependent or equilibrative transporter, lysine uptake was approximately 3-fold faster in sucrose medium compared with NaCl medium. At this low permeant concentration, uptake after 30 min incubation in both solutions was greater than would be

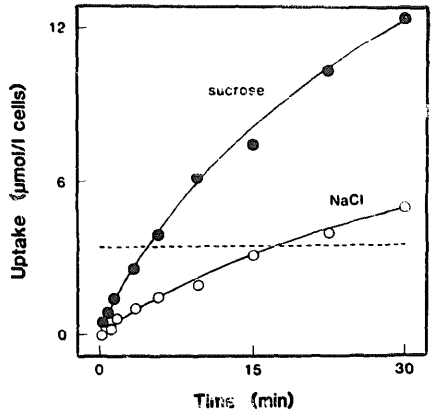

Fig. 1. Time courses of L-lysine uptake by human erythrocytes suspended in isotonic NaCl and sucrose media. Uptake of amino acid (5 μM) at 20°C was measured as detailed in the text. The dashed line represents the uptake expected assuming equilibration of [^{14}C]lysine with intracellular water, calculated on the basis that human erythrocytes contain 70% (v/v) water. Values are means of triplicate determinations.

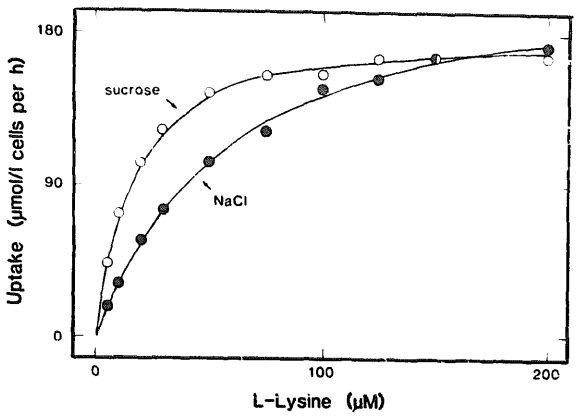

Fig. 2. Concentration-dependence of system y^+-mediated lysine uptake by human erythrocytes suspended in isotonic NaCl and sucrose media. Initial rates of lysine uptake at 20°C, corrected for the arginine-insensitive component of influx, were determined using a 2.5-min incubation period as described in the text. The curves are drawn according to the calculated kinetic parameters listed in Table I. Values are means of triplicate determinations.

expected from equilibration with intracellular water, reflecting system y^+-catalysed homo- and heteroexchange of extracellular [^{14}C]lysine with intracellular non-radioactive dibasic amino acids [3,15]. The same phenomenon was observed subsequently with hagfish erythrocytes (Fig. 6). In subsequent studies reported here for human erythrocytes, we used a 2.5-min incubation period to approximate initial rates of lysine uptake by the cells. In all experiments, system y^+-mediated transport was determined as the difference in [^{14}C]lysine uptake measured in the absence and in the presence of 10 mM extracellular non-radioactive L-arginine. In both NaCl and sucrose media, arginine-insensitive lysine uptake exhibited a linear concentration dependence and accounted for less than 5% of total uptake at the highest lysine concentration studied (200 μM). Previously, it has been established that this residual component of lysine transport is mediated, in part, by Na^+-independent neutral amino acid transport system L [2].

Fig. 2 shows results from a representative experiment comparing the concentration-dependence of system y^+-mediated (arginine-sensitive) lysine uptake (5–200 μM) in NaCl and sucrose media. These curves and the kinetic parameters derived from them by non-linear regression analysis (Table I) are consistent with Na^+ functioning as a competitive inhibitor of the y^+ transporter. Thus, the apparent K_m value for lysine uptake in NaCl medium was 59.4 μM, compared to 15.2 μM in cation-free medium, a ratio of 3.9. In contrast to this large decrease in transporter affinity in the presence of Na^+, lysine uptake in NaCl medium was associated with a modest 1.2-fold higher V_{max}, such that V_{max}/K_m ratios in the two conditions differed by 3.1-fold, a result which agrees quantitatively with the 5 μM lysine uptake data presented in Fig. 1. A similar small difference in V_{max} in NaCl and sucrose media has been observed previously for alanine and lysine transport by horse erythrocyte system asc_1 [8]. This may reflect a non-specific effect of ionic strength on transporter conformation [16] or may be a consequence of the effect of sucrose medium on membrane potential (please see below). A value of 60 μM for the apparent K_m value for lysine uptake by system y^+ in human erythrocytes in NaCl medium is consistent with previously published values at 37°C [2,13].

Specificity of cation inhibition of human erythrocyte system y^+

To investigate further the interaction of Na^+ with the y^+ transporter, we measured the initial rates of 5 μM lysine uptake in media where sucrose was isosmotically replaced by increasing concentrations of NaCl in the range 5–150 mM (Fig. 3). Na^+-inhibition of system y^+ was progressive with respect to Na^+ concentration, with an IC_{50} value in the region of 27 mM. For

TABLE I

Kinetic constants for lysine uptake by human erythrocyte system y^+

Kinetic parameters (±S.E.) for system y^+-mediated lysine uptake (5–200 μM extracellular concentration, 20°C) by cells suspended in isotonic NaCl and sucrose media were determined by non-linear regression analysis of the concentration-dependence data in Fig. 2. The K_m and V_{max} ratios of 3.9 and 1.2, respectively, are both significantly different from 1.0 ($P < 0.01$, Fisher-Behrens test [32]).

	Kinetic parameters		
	Apparent K_m (μM)	V_{max} (μmol/l cells per h)	V_{max}/K_m (h)
NaCl medium	59.4 ± 3.0	228 ± 5	3.8
Sucrose medium	15.2 ± 0.7	183 ± 2	12.0
Ratio	3.9	1.2	0.32

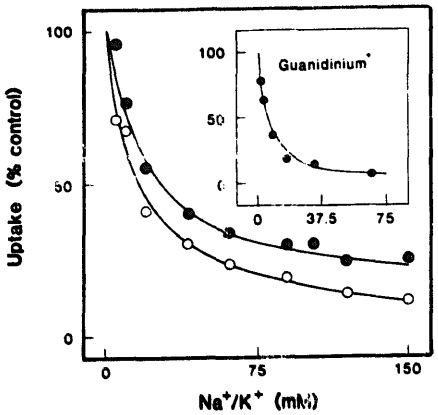

Fig. 3. Na^+, K^+ and guanidinium$^+$ inhibition of lysine uptake by human erythrocyte system y^+. The initial rates of system y^+-mediated lysine uptake (5 μM extracellular concentration, 20°C) were measured in isotonic 300 mM sucrose medium or in media where sucrose was replaced isosmotically with increasing concentrations of Na^+ (●), K^+ (○) or guanidinium (inset). Values are means of triplicate determinations.

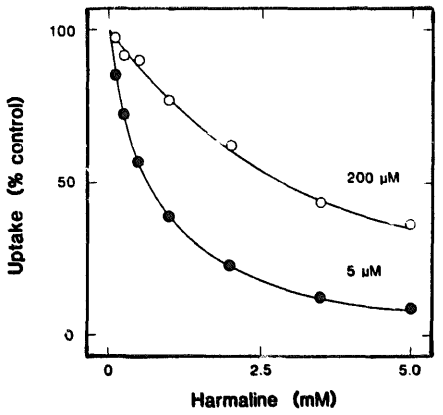

Fig. 4. Harmaline inhibition of lysine uptake by human erythrocyte system y^+. Initial rates of system y^+-mediated lysine uptake at 20°C in sucrose medium were determined at two extracellular lysine concentrations (5 and 200 μM) in the absence (control) or presence of increasing concentrations of harmaline (0.1–5 mM) added to cells simultaneously with permeant. Values are means of triplicate determinations.

comparison, the K_{50} value for Na^+ with respect to L-alanine transport (0.75 mM) by human erythrocyte Na^+-dependent system ASC is 58 mM (Young, J.D. and Fincham, D.A., unpublished data). Fig. 3 also demonstrates that y^+ transport inhibition was not specific for Na^+, sucrose replacement by KCl causing a similar marked reduction in transport activity (IC_{50} 16 mM). Other inorganic (Rb^+ Li^+, Mg^{2+}) and organic cations (choline$^+$, Tris$^+$, guanidinium$^+$), added as their Cl^- salts, were also found to cause inhibition of transport activity, guanidinium being the most effective (78% inhibition at 20 mM guanidinium$^+$) (Table II). A full inhibition curve for this latter cation is presented in the inset to Fig. 3, giving an IC_{50} of 5 mM, a value less than 1/5 that for Na^+. One implication of this series of experiments is that Na^+-substitution by conventional Na^+-replacements would have failed to detect the interaction of y^+ with Na^+. Also, the finding that free guanidinium$^+$ was more potent at inhibiting transporter function than Na^+ or other cations complements the competitive Na^+/lysine interaction seen in Fig. 2 and suggests that the site of Na^+-binding to the transporter is the same as that normally occupied by the amino/guanido group on the dibasic amino acid side chain. Since all cations were present as their Cl^- salts, the Na^+/guanidinium$^+$ and other differences among the effects of various cations (Fig. 3; Table II) excludes the possibility that the observed inhibition of transporter function was anion rather than cation mediated. This distinction is important and has a bearing on the possible role of membrane potential in the response of y^+ to changes in external medium ion composition (please see below).

TABLE II

Inhibition of lysine uptake in human erythrocytes by different inorganic and organic cations

The initial rate of system y^+-mediated lysine uptake (5 μM extracellular concentration) was measured at 20°C in isotonic 300 mM sucrose medium (control) or in media where sucrose was replaced isosmotically by 20 mM of different cations added as their Cl^- salts. Values are means of triplicate determinations. S.E. values were < 1% in all cases (not shown).

Cation	Uptake (% control)
Na^+	55
K^+	46
Rb^+	43
Li^+	49
Mg^{2+}	36
Tris$^+$	63
Choline$^+$	51
Guanidinium$^+$	21

Harmaline inhibition of system y^+ in human erythrocytes

A primary objective of the present study was to investigate the effects of the Na^+-site inhibitor harmaline on y^+ in anticipation that this might also provide information on functional and possible structural similarities between this system and ASC/asc. As shown in Fig. 4, y^+-mediated 5 μM lysine uptake in sucrose medium was inhibited by harmaline with an IC_{50} of 0.6 mM. Raising the permeant concentration to 200 μM increased the IC_{50} to 2.9 mM. This ability of lysine to protect the transporter against harmaline inhibition was investigated formally in an experiment where lysine uptake at 4 different extracellular concentrations (20, 50, 100 and 200 μM) was measured in the absence or in the presence of 1, 2.25 and 3.5 mM harmaline (Fig. 5). As in previous experiments, lysine uptake data were corrected for transport not mediated by system y^+. In contrast to arginine-sensitive lysine transport,

this arginine-insensitive uptake of lysine was unaffected by harmaline concentrations ([I]) up to 5 mM. A double reciprocal plot of the results is shown in Fig. 5A, the pattern of inhibition approximating simple competitive inhibition. The harmaline inhibition constant (K_i) determined from a plot of $1/v$ vs. [I] was 0.23 mM (Fig. 5B). This compares favourably with an apparent K_i value of 0.9 mM for harmaline inhibition of Na^+-binding to human erythrocyte system ASC [8]. The corresponding inhibition constant for harmaline competitive inhibition of lysine transport by horse erythrocyte system asc_1 was 2.6 mM [8]. For asc, the site of harmaline binding to the transporter has been identified is that responsible for binding of the dibasic amino acid side chain positive charge, this site being in the same topographical location as the Na^+-binding site on ASC [8]. To investigate whether Na^+ and harmaline compete for a common binding site on y^+, we determined the apparent K_i value for harmaline inhibition of lysine transport in cells suspended in NaCl medium. As predicted from a common site of binding to the transporter, the presence of Na^+ in the incubation medium increased the apparent K_i value for harmaline inhibition of lysine transport from the previous value of 0.23 mM in sucrose medium to 0.75 mM. This 3.3-fold difference in harmaline K_i closely parallels the 3.9-fold difference in lysine K_m seen in NaCl and sucrose media (Table I).

Effects of membrane potential on lysine transport by human erythrocytes

For human erythrocytes, the conductive permeability for Cl^- is two orders of magnitude higher than for Na^+ and K^+ (see, for example, Ref. 17). Because of this, membrane potential (normally -10 to -14 mV) is determined mainly by the diffusion potential for Cl^- which in turn is determined largely by the transmembrane Cl^- distribution ratio [17]. Dilution or replacement of the external medium with sucrose leads to membrane depolarisation and the establishment of a large positive (inside) membrane potential [18,19]. To investigate the effects of membrane potential (E_m) on system y^+-mediated lysine transport, we compared initial rates of arginine-sensitive lysine uptake (extracellular concentration 7.5 μM) under five conditions: (a) modified NaCl medium (146 mM NaCl, 4 mM KCl, 4 mM Hepes (titrated to pH 7.5 at 20°C with tris base)) ($E_m \approx -10$ mV); (b) KCl medium (150 mM KCl, 4 mM Hepes-Tris) ($E_m \approx -10$ mV); (c) NaCl medium, 1 μM valinomycin ($E_m \approx -66$ mV); (d) KCl medium, 1 μM valinomycin ($E_m \approx +2$ mV); and (e) sucrose medium (300 mM sucrose, 4 mM Hepes-Tris) ($E_m \approx +60$ mV) [20,21].

Results from a representative experiment (mean ± S.E. ($n = 3$)) were 18.9 ± 3.0, 13.9 ± 1.5, 33.0 ± 4.5, 8.1 ± 0.3 and 45.3 ± 7.9 μmol/l cells per h for (a) to (e), respectively. The small difference in lysine uptake between conditions (a) and (b) reflects differential effects of Na^+ and K^+ on the transporter and is consistent with data from Table II and Fig. 3. Since lysine is positively charged at neutral pH, membrane hyperpolarisation ((c) vs. (a)) led to the expected increase in influx rate. Conversely, membrane depolarisation significantly decreased lysine uptake ((d) vs. (b)). These results establish that lysine transport in human erythrocytes is electrogenic and therefore sensitive to changes in membrane potential. However, the effects of membrane potential on transport are *opposite* to those caused by sucrose replacement (e). Because of this secondary effect on membrane potential, replacement

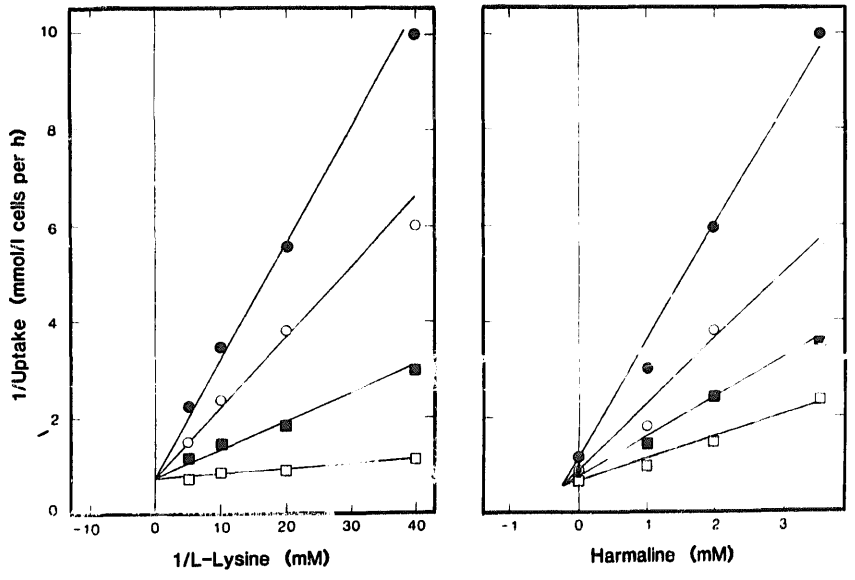

Fig. 5. Kinetic analysis of harmaline inhibition of lysine uptake by human erythrocyte system y^+. Initial rates of system y^+-mediated uptake of lysine (20, 50, 100 and 200 μM) were measured at 20°C in sucrose medium in the absence or in the presence 1, 2.25 and 3.5 mM harmaline. Values are means of triplicate determinations.

of Na^+ by sucrose will underestimate the degree of Na^+-inhibition of lysine transport.

Lysine transport by hagfish erythrocytes

Erythrocytes from the Pacific hagfish (*Eptatretus stouti*), generally regarded as amongst the most primitive of living vertebrates, transport lysine by an arginine-inhibitable y^+-type mechanism, with additional low-affinity uptake by Na^+-independent systems asc and L (Ref. 14 and Young, J.D. and Fincham, D.A., unpublished). In the present context, hagfish erythrocytes differ from human erythrocytes in two important respects. First, cells and plasma are isosmotic with seawater [22]. This means that erythrocytes are normally exposed to substantially higher NaCl concentrations than human erythrocytes ($\approx$ 500 vs. 150 mM NaCl). Second, hagfish erythrocytes are largely deficient in the Cl^-/HCO_3^- exchange transporter [23]. It might therefore be expected, as occurs in *Amphiuma* erythrocytes which also have a low conductive permeability for Cl^- [24], that replacement of extracellular NaCl with ion-free medium will lead to smaller changes in membrane potential than are induced in human cells under the same conditions. For these reasons, and because of the hagfish's primitive nature, we extended the present study to include investigation of the effects of NaCl replacement on lysine transport in this species. In preliminary studies we found that suspension of hagfish erythrocytes in medium containing 1 M sucrose led to cell lysis. We therefore used isosmotic mannitol to substitute for NaCl.

In a first experiment, we measured initial rates of total lysine uptake (15 min incubation, 10°C) in NaCl and isosmotic mannitol media at initial extracellular concentrations of 5 and 200 μM. In agreement with results for human erythrocytes, NaCl replacement by mannitol led to increased rates of lysine uptake, with a larger difference at the lower of the two permeant concentrations (cf. Fig. 2). Uptake rates (μmol/l cells per h [mean $\pm$ S.E. (3)]) were 40 ± 4 and 103 ± 1 in NaCl medium and in mannitol medium, respectively, at 5 μM lysine and 589 ± 20 and 992 ± 126 respectively, at 200 μM lysine. To quantify the effect of NaCl replacement on the y^+ component of lysine transport, we next compared the time course of 5 μM lysine uptake in the two media, measured both in the absence and in the presence of 10 mM nonradioactive arginine. The results presented in Fig. 6 demonstrate a 9.6-fold stimulation of y^+ transport activity upon removal of NaCl from the incubation medium.

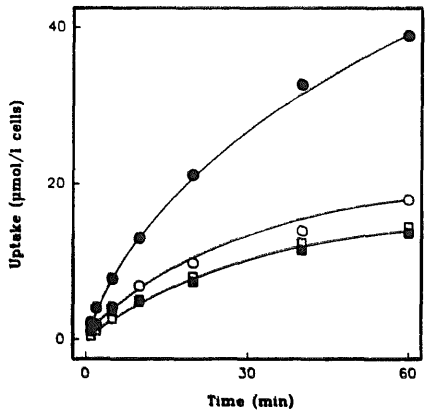

Fig. 6. Time courses of lysine uptake by hagfish erythrocytes suspended in isotonic NaCl (○, □) and mannitol (●, ■) media. Uptake of amino acid (5 μM) at 10°C was measured both in the absence (circles) and in the presence of 10 mM nonradioactive L-arginine (squares) as detailed in the text. Values are means of triplicate determinations.

Discussion

The present series of experiments have demonstrated the ability of dibasic amino acid transport system y^+ from human erythrocytes to function in the almost total absence of extracellular cations. The transporter is not, however, Na^+-independent in the sense that physiological concentrations of extracellular Na^+ cause substantial inhibition of dibasic amino acid influx. At low permeant concentrations, the initial rate of lysine uptake in NaCl medium was 20–30% of that in sucrose medium. A number of observations suggest that this is not a secondary consequence of indirect or nonspecific effects of sucrose substitution on the cells. First, Na^+-inhibition of lysine transport was competitive. Second, we have shown in earlier studies that the same medium substitution has no significant effect on the apparent K_m for lysine uptake by system asc_l in horse erythrocytes [8]. Third, we have established that lysine transport in human erythrocytes is electrogenic, but have demonstrated that the effects of membrane potential on lysine transport are opposite to those caused by sucrose replacement. We therefore interpret our present results as a direct demonstration of Na^+-binding to system y^+. In other experiments, we also observed similar results with y^+ in erythrocytes from a primitive vertebrate, the hagfish, indicating that Na^+-inhibition is a general property of this class of amino acid transporter. The magnitude of the response was greater in hagfish than in human erythrocytes. This may partly reflect the higher Na^+ concentration in hagfish medium (500 mM) and also larger effects of NaCl removal on the membrane potential of human erythrocytes.

Previously, it has been established that y^+ from a variety of sources can interact with neutral amino acids when Na^+ is also present [3,9–12]. Two types of neutral amino acid interaction with system y^+ have been documented. First, neutral amino acids have been shown to inhibit dibasic amino acid uptake, but only when Na^+ or other appropriate ion is present in the extracellular medium [9–12]. Second, intracellular neu-

tral amino acids have been reported to stimulate the influx of dibasic amino acids, this effect being abolished in Na^+-depleted cells [3]. The ability of Na^+ to function directly as an effective inhibitor of system y^+ in the absence of neutral amino acid has not previously been observed. We attribute this to the widespread use of choline chloride as NaCl substitute (see, for example, Refs. 3, 10–12, 25). As shown in Table 2, choline$^+$ and Na^+ are equally effective inhibitors of human erythrocyte y^+. As an example of this, Christensen and co-workers observed a small amount of Na^+-inhibition ($\approx 6\%$) of homoarginine exchange in Ehrlich ascites tumour cells (Fig. 8, Ref. 11). It is likely that the effect would have been larger if sucrose had been used in place of choline chloride. Although at the limit of detection, the same study reported an increase in $^{22}Na^+$ entry into cells when the combination of neutral amino acid and Na^+ stimulated dibasic amino acid exodus. In the absence of extracellular amino acids, homoarginine exodus was substantially greater in NaCl medium than in choline chloride (see also Fig. 8, Ref. 11). Thus, although system y^+ seems to be inhibited equally well by both Na^+ and choline$^+$, it may transport Na^+ much better than it transports choline$^+$.

In our experiments, inhibition of system y^+ by Na^+ was competitive with respect to lysine concentration, indicating that the site of cation binding is within the permeant translocation site of the transporter, a suggestion also made by Christensen et al. [11] on the basis of their homoarginine exchange experiments. Guanidinium$^+$ was the most effective cation inhibitor of y^+ (Table II, Fig. 3), indicating that cations bind to the y^+ translocation site at a location normally occupied by the dibasic amino acid side chain positive charge. Our results therefore provide new evidence in support of the original postulate of Christensen and Antonioli [9] that the y^+ translocation site recognises the combination (Na^+ + neutral amino acid) as being equivalent to dibasic amino acid. In most physiological situations, extracellular cation concentrations are unlikely to vary to an extent sufficient to cause detectable changes in cellular dibasic amino acid permeability. However, as reviewed by White [3], Na^+-dependent transport via y^+ may be a significant route of neutral amino acid permeation in a number of cell types.

The relative effectiveness of different cations in potentiating the inhibitory actions of neutral amino acids on dibasic amino acid transport by y^+ depends both on the cell type and on the structure of the neutral amino acid. In agreement with the broad specificity of cation binding to human erythrocyte y^+ seen in the present study (Table II), Na^+, Li, K + and Rb^+ have been shown to be effective in potentiating straight chain apolar neutral amino acid inhibition of arginine uptake by rabbit reticulocytes [12]. In the presence of a hydroxyl group on carbon 4, Na^+ and K^+ are much more effective than Li^+ or Rb^+, while choline$^+$ is ineffective. For system y^+ in Ehrlich ascites cells, only Na^+ and Li^+ enhance the inhibitory actions of apolar and hydroxy neutral amino acids [12]. For the latter category of neutral amino acids, it has been postulated that binding of the amino acid to the translocation site is enhanced by direct co-substrate binding between Na^+ (or other cation) and the side chain hydroxyl group. The extent to which different neutral amino acids modify the affinity and selectivity of cation binding to human erythrocyte y^+ reported here remains to be established.

In addition to demonstrating cation inhibition of y^+, our investigations have also established that the hallucinogenic alkaloid harmaline binds competitively with Na^+ to the y^+ translocation site in a manner directly analogous to its interactions with amino acid transport systems ASC and asc [8]. As detailed in the Introduction, Na^+-dependent system ASC and Na^+-independent system asc exhibit a common selectivity for small neutral amino acids of intermediate size and both possess specific recognition sites for side chain amino and guanido groups, these recognition sites permitting the transporters to interact with dibasic amino acids. For ASC, this recognition site is the site of Na^+ and harmaline binding to the transporter. Binding constants for Na^+ and harmaline at this site are within the same range as those for binding to the same topographical site on y^+ The ASC site also accepts free guanidinium$^+$ [6]. The equivalent recognition site on asc binds harmaline with a similar affinity as ASC and y^+, but exhibited no detectable interaction with Na^+ when the kinetics of lysine (and alanine) transport were compared in NaCl and sucrose media [8]. Interestingly, the latter study was performed in the presence of 15 mM tris HCl. This concentration of tris$^+$ would be sufficient to cause significant inhibition of y^+ (Table II) and might conceivably mask a small Na^+-interaction with asc. We are presently investigating this possibility.

In conclusion, ASC, asc and y^+ represent a spectrum of closely related transport activities. We interpret these similarities as evidence of a common evolutionary origin with the implication of significant structural homology at the primiary sequence level. From a structural standpoint, it is likely that only minor changes in permeation site configuration would be required to generate the various transport and other functional properties of the three systems. Since ASC funcitons as a classical Na^+-coupled system, these conclusions have interesting implications with respect to the evolution and mechanism of Na^+-coupling in secondary active transporters. ASC and y^+ are particularly important in this regard because of the evidence that substrate and Na^+ bind in close juxtaposition to each other. This contrasts with the intestinal Na^+-dependent glucose transporter where the Na^+ and permeant binding sites

appear widely separated [26]. The mechanism of cation coupling in ASC/y^+ is also probably different from the anion-dependent amino acid transporters Gly and β, which in some occurrences exhibit a 2:1 Na^+/amino acid coupling ratio [27,28].

Finally, we draw attention to recent studies by Van Winkle and co-workers [29,30] who have shown similar cation and harmaline interactions with another novel Na^+-independent amino acid transport system $b^{0,+}$ from preimplantation mouse blastocysts. This transporter exhibits a high affinity for both lysine and leucine and interacts only weakly with alanine [31]. Clearly, it will be instructive to study harmaline and cation effects on other 'Na^+-independent' amino acid transporters.

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