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Arginine uptake through a novel cationic amino acid:K^+ symporter, System R^+, in brush border membrane vesicles from larval *Manduca sexta* midgut

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Abstract

A concentrative uptake of arginine into brush border membrane vesicles (BBMV) from the midgut of *Manduca sexta* larvae was driven by an inwardly directed K^+ gradient. The pH-dependence of the initial rate of arginine uptake between pH 7 and 10.5 paralleled the titration curve of the amino acid, suggesting that cationic arginine is the principal ionic form that is transported. In the presence of K^+, at pH 7.4, arginine uptake was *cis*-inhibited and *trans*-stimulated by arginine and lysine but not by any other naturally occurring amino acids; it was also *cis*-inhibited by homoarginine and ornithine. Taken together, these data argue that arginine, lysine and their analogues share a cationic amino acid:K^+ symporter (cotransporter), which we will designate as System R^+. This novel symporter has a substrate spectrum similar to that of the uniporter, System y^+, in that it accepts arginine$^+$, lysine$^+$, homoarginine$^+$ and ornithine$^+$ and rejects histidine. However, it differs from y^+ in that it is cation-dependent and is almost inactive at pH 5.5.

Keywords: Lepidopteran larva; Cotransport; Transporter

1. Introduction

Arginine, a basic amino acid in proteins, is also a component of the urea cycle and is a precursor of the vertebrate phosphagen (creatine phosphate), the invertebrate phosphagen (arginine phosphate) [1] and nitric oxide [2]. Yet, in most animals, arginine is an essential amino acid, i.e. its synthesis is insufficient for growth and the shortfall must be made up by transport across the intestinal epithelia. In vertebrates, Na^+-independent and Na^+-dependent systems both appear to mediate the uptake of arginine and other basic amino acids. The Na^+-independent uptake of cationic arginine and lysine is mediated in non-epithelial and epithelial cells by System y^+ [3]. The Na^+-dependent uptake of lysine has been studied in brush border membrane vesicles (BBMV) prepared from high-protein-diet rat intestine [4], rat kidney [5] and eel intestine [6]. K^+-dependent lysine uptake has been described in lepidopteran midgut BBMV [7]. Although the cation-independent System y^+ is well characterized [8], the cation-dependent systems are not well understood, particularly with respect to arginine uptake.

One complication in analysing basic amino acid uptake is that the ionic form changes with pH [9]; thus, arginine and lysine are primarily cationic at pH 7 but largely zwitterionic at pH 10. Another complication is that the concentrative uptake of cationic amino acids can be driven either by a co-substrate-ion concentration gradient or by an inside-negative voltage gradient. Implicitly, two types of carrier may be involved: a postulated symporter or a uniporter such as System y^+.

Amino acid uptake by the lepidopteran midgut has several unusual properties that promise new insight into these complications. Lepidopteran larvae are phytophagous and have a highly alkaline midgut, with pH values ranging from > 11 in anterior-middle midgut to < 8 in posterior midgut [10]; amino acid uptake has been characterised in BBMV from both midgut regions [11]. The midgut alkalinity is thought to be an adaptation to the high tannin content of ingested leaves in that it aids their digestion and absorption [12]. The physiological co-substrate for symport is K^+

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rather than Na^+ [13]. Moreover, the midgut epithelial cells lack Na^+/K^+ ATPase [14]; instead the brush border membrane is energized by an H^+ translocating V-ATPase in parallel with an K^+/H^+ antiporter [15]. Finally, the symport in vivo is driven by the large transmembrane voltage ($\Delta\Psi \approx 240$ mV, cytoplasm negative), the $[K^+]$ being > 100 mM in both cell and lumen but there being little or no transmembrane K^+ activity gradient [16].

In lepidopteran midgut, total arginine uptake (in all ionic forms) faces a 5-fold, adversely-directed (cell to lumen) concentration gradient [17]; in the highly alkaline anterior-middle midgut regions, the concentration gradient of cationic arginine can be 50-fold. Consequently, arginine symport is favored in the less alkaline, posterior region of the midgut. Although a highly energized membrane would be necessary for arginine uptake in anterior-middle midgut, the in vivo transapical voltage difference of −240 mV would be more than adequate for this purpose.

We describe here a new, intestinal, arginine and lysine:K^+ or Na^+ symporter in brush border membrane vesicles from *M. sexta*. The essential property of this novel system is that, although it recognizes both arginine and lysine [18], it takes up arginine and not lysine when both basic amino acids are present simultaneously (Liu and Harvey, unpublished data); i.e. this system appears to be designed for arginine uptake. After discussions with Dr. Halvor Christensen and others, we designate this new symporter, System R^+, following the rules set for system naming [9,19]: "R" is the single letter symbol for arginine, upper case "R" denotes the symporter's K^+ or Na^+ dependence and superscript "+" indicates that it recognizes only the cationic forms of arginine and lysine. In this paper we characterize arginine uptake by System R^+ in midgut BBMV and show that this system probably accounts for the "lysine-specific" transporter previously identified in *Philosamia cynthia* [7]; we also show that zwitterionic arginine uptake by System B is weak in these vesicles. In a subsequent paper we will show that lysine uptake is mediated both by System R^+ and by well-known zwitterionic systems [18]; uptake kinetics will be compared in a third paper.

2. Materials and methods

2.1. Amino acid uptake measurements

Preparation of vesicles, establishment of intravesicular component concentrations and uptake measurements were similar to those described previously [20]. Midguts were isolated from fifth instar *M. sexta* larvae in ice cold MET buffer (mannitol 300 mM, EDTA 5 mM, Tris 17 mM, pH 7.4). BBMV were prepared from freshly isolated midguts by a differential magnesium precipitation method [21–23]. Aliquots of BBMV preparation that were frozen rapidly, by plunging the vial into liquid nitrogen, and thawed rapidly, by shaking the vial in tap water, gave uptake values identical to those used immediately (e.g. 0.55 ± 0.026 nmol L-[^{3}H]Lys mg protein^{-1} s^{-1} for fresh preparations, vs 0.55 ± 0.0062 units for frozen preparations; also see [24]). Protein concentrations were determined by the dye-protein binding method [25] using a kit from BioRad (Richmond, CA) with bovine serum albumin as a standard. Intravesicular and extravesicular component concentrations are given in each figure legend as "inside" and "outside" respectively. Amino acid uptake was measured with radioactive tracers at 25 ± 1°C in triplicate or quadruplicate by a rapid filtration technique [23,26]. The initial uptake rate was calculated as the mean slope ± SD of lines through 3 or 4 pairs of uptake values measured at 2 and 6 s after mixing, using the computer program, SigmaPlot (Jandel Scientific, San Rafael, CA).

2.2. Chemical reagents

L-[2,4-^{3}H]Arginine was from ICN Biochemicals (Costa Mesa, CA) or from Sigma Chemical Co. (St. Louis, MO). L-Homoarginine was from INC Biochemicals (Aurora, OH). L-Ornithine · HCl was from Aldrich Chemical Company (Milwaukee, WI). Non-radioactive amino acids, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), tris[hydroxymethyl]amino-methane (Tris), car-

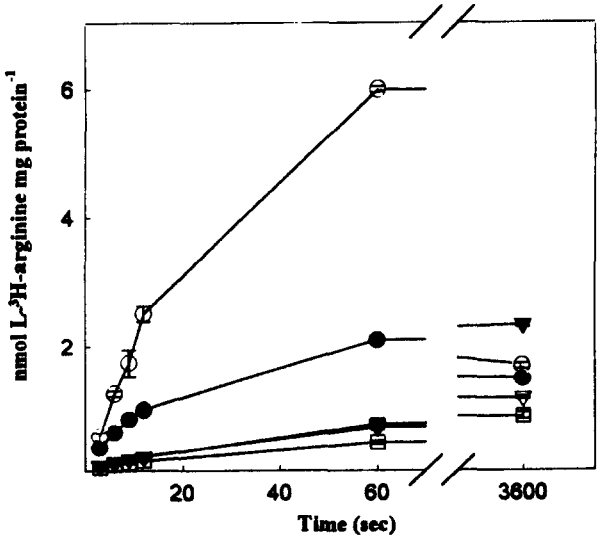

Fig. 1. Potassium gradient-dependent arginine uptake. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 400, AMPD 50, (outside) KSCN 100, mannitol 200, AMPD 50 (–○–); (inside) $TMANO_3$ 200, AMPD 50, (outside) KNO_3 100, $TMANO_3$ 100, AMPD 50 (–●–); (inside) mannitol 400, AMPD 50, (outside) $TMANO_3$ 100, mannitol 200, AMPD 50 (–▼–); (inside) mannitol 200, AMPD 100, (outside) mannitol 200, AMPD 100, 0.1 FCCP added to vesicle suspension 30 min before experiment (–▽–); (inside and outside the same) mannitol 400, AMPD 50 (–□–); $pH_i = pH_o = 10.0$ in (–○–) (–●–) (–▼–) (–□–); $pH_i = 8.0$ and $pH_o = 9.3$ in (–▽–); pHs were adjusted with HCl. In each of the five conditions 0.5 mM L-[^{3}H]arginine was present outside of the vesicles.

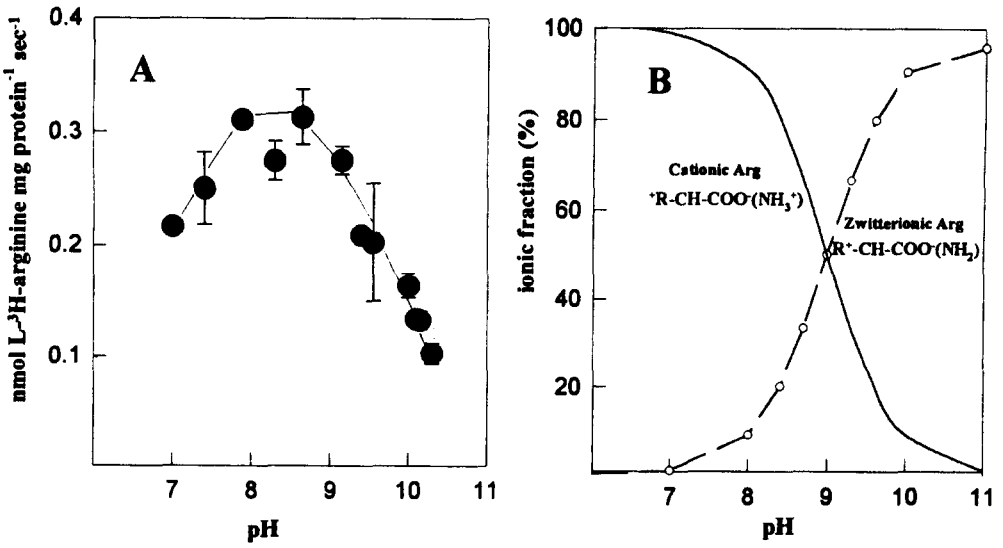

Fig. 2. Effects of alkaline pH on arginine uptake and ionic form. (A) pH profile of arginine uptake. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 185, Hepes 10, Tris 5, pH 7.4; (outside) mannitol 117, KSCN 50, Tris-MES 30 from pH 7.0 to 8.3, AMPD-MES 30 from pH 8.5 to 9.0, AMPD 30 from pH 9.2 to 10.2, L-[^{3}H]arginine 0.04. (B) Relative concentrations of cationic and zwitterionic arginine as a function of pH. Values in the plot were calculated from pK 9.0 of the α-amino group.

bonyl cyanide *p*-(trifluoromethoxy)phenyl-hydrazone (FCCP) and tetramethylammonium hydroxide (TMAOH) were from Sigma. Aminomethylpropanediol (AMPD) was from the Eastman Kodak Company (Rochester, NY). 2-(*N*-morpholino)ethanesulfonic acid (MES) was from ICN Biochemicals (Cleveland, OH). All other reagents were analytical grade products from either Fisher (Pittsburgh, PA) or Mallinckrodt (St. Louis, MO).

3. Results

3.1. Concentrative arginine uptake is driven by a K^+ gradient

L-[^{3}H]Arginine (0.5 mM) uptake into vesicles was measured at pH 10.0 in the presence of inwardly directed gradients of (1) 100 mM KSCN, (2) 100 mM K^+ (outside

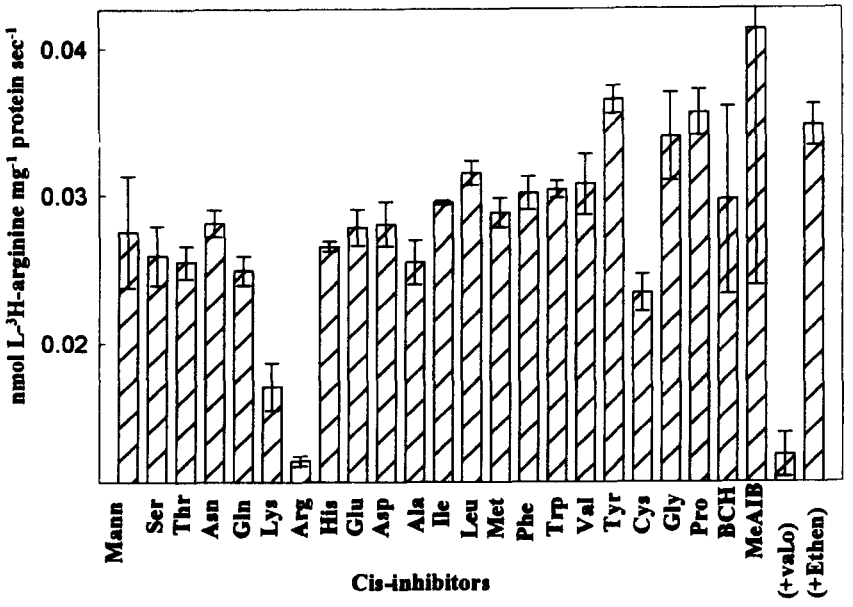

Fig. 3. *cis*-Inhibition of arginine uptake by 20 amino acids and two model substrates, BCH and MeAIB. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 400, Hepes 90, Tris 45, pH 7.4, (outside) mannitol 200, KCl 100, Hepes 90, Tris 45, pH 7.4, L-[^{3}H]arginine 0.1, inhibitor amino acid, BCH or MeAIB 10. For the column labeled "valino", valinomycin dissolved in ethanol was added to the vesicle suspension one hour before the experiment to yield a final concentration of 4 μg mg^{-1} protein. The ethanol control contained the same amount of ethanol alone as that contained in the vesicle suspension.

100 mM KNO_3, inside 100 mM $TMANO_3$ [tetramethylammonium nitrate]), (3) 100 mM $TMANO_3$, and (4) a mannitol control. It was also measured in the absence of K^+ with an imposed $\Delta\Psi$ (inside negative) by setting $pH_i = 8.0$ and $pH_o = 9.3$ with FCCP present (Fig. 1). In the presence of a K^+ gradient, the 1 min uptake value was much higher than the 60 min equilibrium value, suggesting that arginine uptake in *M. sexta* is mediated by amino acid:K^+ symport. In the absence of K^+ the imposed $\Delta\Psi$ alone did not drive arginine uptake suggesting that the uptake is not mediated by uniport.

3.2. Arginine's cationic form is selected by the symporter

The initial rate of arginine:K^+ symport is bell-shaped between pH 7 and 10.5 (Fig. 2A), closely paralleling the pH-dependence of the cationic fraction (Fig. 2B). The uptake rate decreases at lower pH (data not shown), probably due to titration of charges on the carrier protein. The optimum pH for cationic arginine uptake is ca. 8 (Fig. 2), which is the physiological pH of the posterior midgut [10].

3.3. Arginine symport at pH 7.4 is cis-inhibited by lysine, arginine, homoarginine and ornithine

Arginine uptake was measured at pH 7.4, where it is largely cationic, in separate experiments with each of the 20 naturally occurring amino acids, as well as α-methyl-amino-isobutyric acid (MeAIB, a System A model substrate [9]) and 2-amino-2-norbornane-carboxylic acid hemihydrate (BCH, a System L model substrate [9]) (Fig. 3). The extravesicular concentration of all of the inhibitory amino acids was 10 mM except for cystine which was < 10 mM due to its low solubility. Arginine and lysine inhibited 90% and 60% of arginine uptake, respectively. By contrast, none of the other eighteen natural amino acids or the two model substrates inhibited arginine uptake substantially. When the vesicle suspension was preincubated with the K^+ ionophore, valinomycin, the uptake was virtually abolished. Ethanol at the same concentration that was used to dissolve valinomycin had no effect on uptake.

3.4. Concentrative arginine uptake is trans-stimulated by lysine and arginine

The time courses of *trans*-stimulation of arginine uptake by lysine, arginine, leucine and mannitol were measured at pH 7.4 (Fig. 4) and 10.0 (Fig. 5). The elicitor concentration inside the vesicles was 40 mM in each case and 50 mM K^+ was present both inside and outside the vesicles; valinomycin was present to eliminate $\Delta\Psi$. Thus the vesicles were virtually isosmotic with respect to the external solution and the only gradients present were those of the outwardly directed elicitor and the inwardly directed, labeled arginine. Both lysine and arginine elicited arginine uptake above equilibrium values at both pH val-

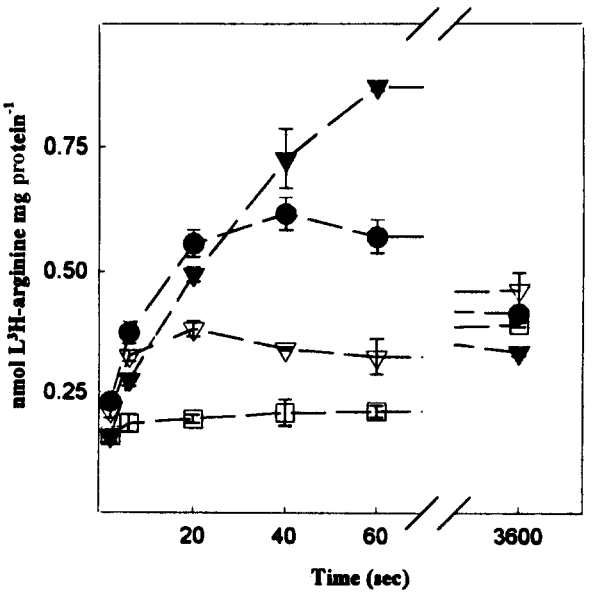

Fig. 4. Time course of *trans*-stimulation of arginine uptake by lysine, arginine, leucine and mannitol at pH 7.4. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 80, KSCN 50, Hepes 90, Tris 45, pH 7.4, elicitor amino acid: lysine (–●–) arginine (–▼–) leucine (–▽–) mannitol (–□–) 40, (outside) mannitol 120, KSCN 50, Hepes 90, Tris 45, pH 7.4, elicitor amino acid 2.0, L-[^{3}H]arginine 0.1. One hour before the experiment valinomycin dissolved in ethanol was added to the vesicle suspension to yield a final concentration of 8 μg mg^{-1} protein.

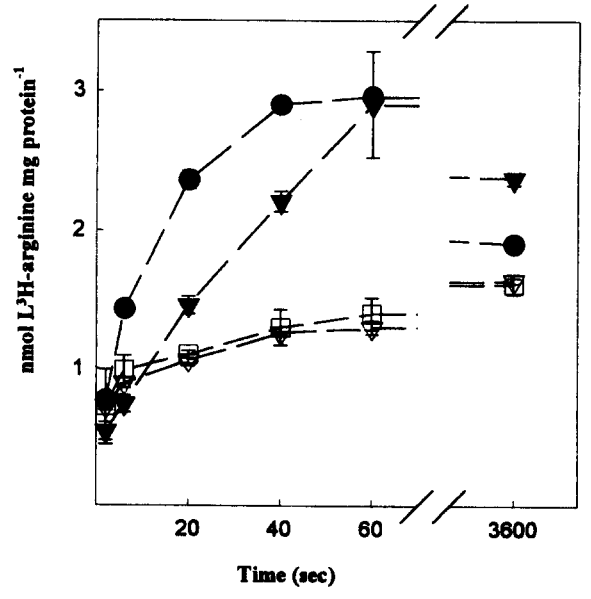

Fig. 5. Time course of *trans*-stimulation of arginine uptake by lysine, arginine, leucine and mannitol at pH 10.0. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 80, KCl 100, AMPD 50, pH 10.0, elicitor amino acid: lysine (–●–) arginine (–▼–) leucine (–▽–) mannitol (–□–) 40, (outside) mannitol 120, KCl 100, AMPD 50, pH 7.4, elicitor amino acid 1.0, L-[^{3}H]arginine 0.2. One hour before the experiment valinomycin dissolved in ethanol was added to the vesicle suspension to yield a final concentration of 8 μg mg^{-1} protein.

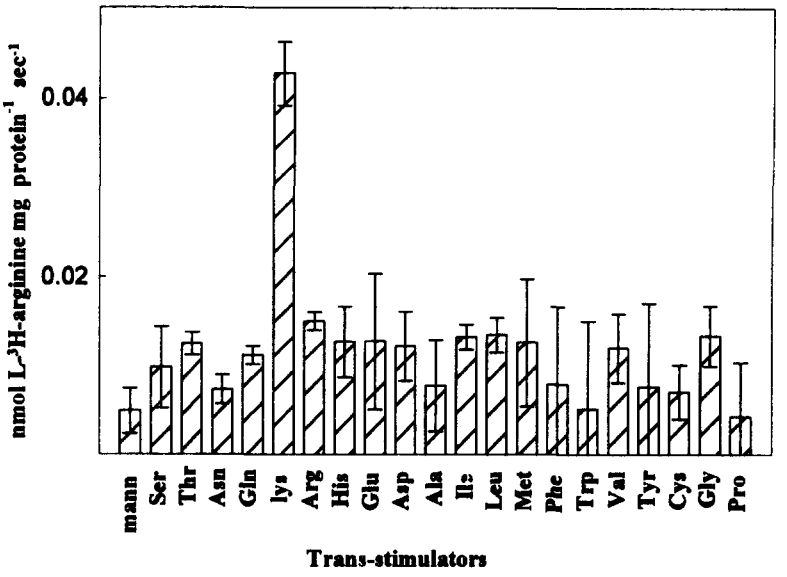

Fig. 6. *trans*-stimulation of arginine uptake by 20 common amino acids at pH 10.0. The final concentrations (mM) of components inside and outside of the vesicles at time zero of incubation were: (inside) mannitol 100, KSCN 50, AMPD 50 pH 10.0, elicitor amino acid 20, (outside) mannitol 120, KSCN 50, AMPD 50, pH 10.0, elicitor amino acid 1.0, L-[^{3}H]arginine 0.1. One hour before the experiment valinomycin dissolved in ethanol was added to the vesicle suspension to yield a final concentration of 8 μg mg^{-1} protein.

ues in contrast to leucine and mannitol which did not do so; however, leucine did stimulate the initial arginine uptake rate (Figs. 4 and 5). Comparison of the initial rates of arginine uptake at pH 10, that had been *trans*-elicited by 20 mM of the tested amino acids in the absence of a cation gradient (Fig. 6), reveals that lysine strongly enhanced the uptake rate whereas arginine and a dozen other amino acids enhanced it but weakly.

4. Discussion

4.1. Arginine uptake is not mediated by a uniporter

In the presence of K^+, at pH 7.4 – where it is largely cationic – arginine uptake increased in response to an inside negative potential difference induced by an inwardly directed SCN^- gradient (Fig. 1). However, in the absence of K^+, arginine uptake no longer responded appreciably to the SCN^- gradient, an inwardly directed NO_3^- gradient or an outwardly directed H^+ gradient in the presence of FCCP (Fig. 1). These results suggest that a uniporter does not mediate the transport of arginine into the vesicles.

4.2. Arginine uptake by System B is weak

The ionic form of substrate amino acids has long been recognized to be a key factor in substrate recognition by cotransporter proteins [9,27,28]. At pH 10, where it is largely zwitterionic, arginine uptake is accelerated strongly only by lysine (Fig. 6). Although many neutral amino acids (zwitterionic at this pH) elicit lysine uptake [18], leucine does not elicit arginine accumulations (Fig. 5). However, leucine appears to be better than mannitol at eliciting the initial uptake rate of arginine at pH 10 (Figs. 4 and 6), suggesting that zwitterionic arginine and leucine may share System B. Although Hennigan et al. [29] showed that arginine failed to promote leucine, alanine or phenylalanine accumulation, the stimulation of arginine initial uptake by leucine (Figs. 4 and 5) and the low level *trans*-stimulation of arginine uptake by several amino acids at pH 10 (Fig. 6) caution against ruling out zwitterionic arginine uptake by System B.

4.3. Arginine uptake at pH 7.4 is mediated by a cationic amino acid / K^+ symporter (System R^+)

The pH profile of arginine's initial uptake rate (Fig. 2A) and the titration curve of its cationic form (Fig. 2B) are virtually congruent. The decrease in initial uptake rate, as the pH is increased from 8.5 to 10.0, parallels the progressive decrease in availability of arginine's cationic form, suggesting that the symporter requires cationic arginine. Furthermore, only lysine and arginine effectively *cis*-inhibit arginine uptake at pH 7.4. These amino acids could also elicit arginine accumulations at pH 7.4, whereas leucine, a favorite System B substrate, could not do so (Fig. 4). These results suggest that System R^+ transports only cationic arginine and lysine, along with their homologues, homoarginine and ornithine. This substrate spectrum is similar to that of System y^+, the widely distributed cationic amino acid uniporter [30–32].

4.4. The novel System R^+ accounts for the "lys specific" symporter

A large number of amino acid transport systems have been identified in the plasma membranes of non-epithelial and epithelial cells of mammals [33–36]. Several amino acid:cation symporters have been reported in BBMV from the midgut of the lepidopteran larvae, *P. cynthia* [7,37] and *M. sexta* [29]. Although most of these insect symporters resemble mammalian ones, a lysine specific symporter that does not interact with arginine [7] was reported. This report was unexpected because the basic amino acid systems of both invertebrate and vertebrate epithelial and nonepithelial cells recognize arginine as well as lysine [7]. However, in the *P. cynthia* study, the effects of lysine, arginine and various other amino acids were investigated only on lysine uptake but not on arginine uptake. When we examined the effects of lysine and arginine on arginine uptake we found that they share a transport system in BBMV from insects just as they do in BBMV from mammals [6].

4.5. System R^+ differs from System y^+L

System R^+ has a superficial resemblence to the novel System y^+L from human erythrocytes and placenta [38] in that both systems recognize cationic amino acids, but System R^+ requires K^+ or Na^+ for cationic amino acid uptake.

4.6. System R^+ and System y^+ may have a common ancestor

The novel System R^+, like the well-known System y^+ [8,30], accepts cationic arginine, lysine, homoarginine and ornithine and rejects histidine (Figs. 4 and 6), but unlike System y^+ it is severely depressed at pH 5.5. Also unlike System y^+, the presence of (surrogate) K^+ or Na^+ does not render it inhibitable by neutral amino acids. System R^+ is a cationic arginine or lysine:K^+ symporter rather than being a uniporter like System y^+. Whether or not the two systems are genetically related can be determined as soon as a cDNA encoding the symporter is cloned and sequenced.

5. Conclusions

A novel system, R^+, has been identified in BBMV from the midgut of larval *M. sexta*. System R^+ recognizes only the cationic forms of arginine and lysine. Either a K^+ or Na^+ gradient can drive the symport in vesicles but K^+ is the symporting cation in vivo. System R^+ functions above pH 5.5, with an optimum pH slightly greater than 8, the pH of posterior midgut in vivo.

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