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Substrate Selectivity and pH Dependence of KAAT1 Expressed in *Xenopus laevis* Oocytes

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Abstract. When expressed in *Xenopus* oocytes KAAT1 increases tenfold the transport of L-leucine. Substitution of NaCl with 100 mM LiCl, RbCl or KCl allows a reduced but significant activation of L-leucine uptakes. Chloride-dependence is not strict since other pseudohalide anions such as thyocyanate are accepted. KAAT1 is highly sensitive to pH. It can transport L-leucine at pH 5.5 and 8, but the maximum uptake has been observed at pH 10, near to the physiological pH value, when amino and carboxylic groups are both deprotonated. The pH value mainly influences the V_{max} in Na^+ activation curves and L-leucine kinetics. The kinetic parameters are $K_{mNa} = 4.6 \pm 2$ mM, $V_{maxNa} = 14.8 \pm 1.7$ pmol/oocyte/5 min for pH 8.0 and $K_{mNa} = 2.8 \pm 0.7$ mM, $V_{maxNa} = 31.3 \pm 1.9$ pmol/oocyte/5 min for pH 10.0. The kinetic parameters of L-leucine uptake are: $K_m = 120.4 \pm 24.2$ μM, $V_{max} = 23.2 \pm 1.4$ pmol/oocyte/5 min at pH 8.0 and $K_m = 81.3 \pm 24.2$ μM, $V_{max} = 65.6 \pm 3.9$ pmol/oocyte/5 min at pH 10.0.

On the basis of inhibition experiments, the structural features required for KAAT1 substrates are: (i) a carboxylic group, (ii) an unsubstituted α-amino group, (iii) the side chain is unnecessary, if present it should be uncharged regardless of length and ramification.

Key words: Amino acid transport — Cotransport — KAAT1 — Inhibition — *Xenopus laevis* oocyte

Introduction

Over the past few decades amino acid absorption has been studied by means of many different experimental approaches involving whole organs, epithelial tissues, isolated cells, purified membrane vesicles and reconstituted proteoliposomes. From the first studies, it was apparent that several transporters were simultaneously active in a tissue and also in a single cell. Therefore many studies were addressed towards discriminating between an increasing number of transport systems with overlapping substrate specificities.

A clear discrimination was made between Na^+-dependent and Na^+-independent systems: the former being able to transduce the energy stored in the Na^+ gradient for concentrative amino acid uptake (cotransporter), the latter being involved in passive facilitated amino acid uptake (uniporter). Concentrative amino acid uptake can also occur by H^+-coupled transport, by exchange mechanisms with other amino acids, or simply by the energization of the membrane electrical potential (Castagna et al., 1997). One of the most particular amino acid cotransport mechanisms was found in the midgut of lepidopteran larvae where K^+-coupled amino acid transporters are expressed (Giordana et al., 1982).

In recent years, the molecular identification of some amino acid transport proteins allowed the correlation of structure and function and the unambiguous assessment of some physiological features to a particular protein (Palacin et al., 1998). The first K^+-coupled neutral amino acid cotransporter (KAAT1) has recently been cloned (Castagna et al., 1998) from a larval lepidopteran midgut cDNA library (*Manduca sexta*). This cotransporter is expressed in the brush-border apical membrane of absorptive cells and, to match the peculiar ionic conditions of this tissue, it accepts as driver cations both potassium and sodium, the electrochemical gradients of which favor their movement from lumen to cells. KAAT1 cDNA encodes a 634-aa residue protein with a modest (38%) identity with amino acid transporters belonging to the Na^+- and Cl^--dependent GABA transporter superfamily.

The epithelial tissue lining the large gut of leaf-eating lepidopteran larvae generates high electrical po-

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tentials and steep ionic gradients between lumen and cells. The functional unit of this tissue is made up of a goblet cell surrounded by about five electrically coupled columnar cells. A proton V-ATPase located in the apical membrane of goblet cells generates a high transmucosal membrane potential, lumen positive, which energizes a $2H^+/K^+$ antiporter in goblet cells and a K^+-dependent amino acid uptake in columnar cells. The antiporter mediates the potassium secretion and contributes to the alkalinization of the lumen, where pH can reach extremely high values (Wieczorek et al., 1989; Dow, 1992; Harvey & Wieczorek, 1997; Harvey et al., 1998).

Since KAAT1 is a transporter characterized by a broad specificity both for ions and cotransported amino acids, the aim of this study is to analyze the structural features required for KAAT1 substrates, determine the kinetic parameters of leucine transport and study the dependence of amino acid transport on extracellular pH, using determinations of radio labeled amino acid uptake in KAAT1 cRNA-injected *Xenopus laevis* oocytes.

Materials and Methods

Oocyte Expression of KAAT1

KAAT1 cRNA was obtained by in vitro transcription using T7 RNA polymerase (Stratagene) after linearization by *Not1* (Gibco) of the construct p-SPORT1 KAAT1-cDNA. Plasmid extraction was performed using QIAGEN kit.

After isolation from *Xenopus laevis*, oocytes were defolliculated with 1 mg/ml of collagenase A (Boehringer Mannheim) in a Ca^{2+}-free buffer OR II (in mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES/Tris pH 7.5 for 1 hr at room temperature. Mature (stage V–VI) and healthy defolliculated oocytes were selected and maintained at 16.5°C in Barth's medium (in mM): 88 NaCl, 1 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, 10 HEPES/Tris pH 7.5 with the addition of 50 mg/l gentamycin sulfate. On the following day, oocytes were injected with the synthesized cRNA (12.5 ng/oocyte) or with water (50 nl) as the control, using the Drummond injection system.

Transport Experiments

The uptake of 0.1 mM L-[^{3}H]leucine and other 0.1 mM radiolabeled compounds ([^{3}H]glycine, L-[^{3}H]lysine, L-[^{3}H]glutamic acid, [^{14}C]α-methyl-D-glucopyranoside and D-[1-^{14}C]mannitol) (200–500 KBq/ml, Amersham-Pharmacia-Biotech) was measured 3 days after injection. Groups of 8–10 oocytes were incubated in 100 μl of uptake solution (in mM): 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/Tris pH 8.0. For the uptake with varied Na^+ concentration, NaCl was replaced totally or partially by choline-Cl. Unless otherwise indicated, transport experiments were performed at pH 8.0. For pH-dependence experiments, HEPES/Tris was used in the range 7–9 to prepare uptake solutions with varied pH, whereas MES (2-(N-morpholino)ethanesulfonic acid), CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) and CAPS (3-cyclohexylamino)-1-propanesulfonic acid) were used for pH 5.5, 9.5 and 10.0 respectively. To minimize oocytes damage, uptakes at extreme pH values were performed after 5 min of incubation.

The data reported in most figures are the mean of KAAT1-mediated transport of several independent experiments, unless otherwise indicated. KAAT1-mediated transport represents the difference between the mean uptake measured in cRNA-injected oocytes and the mean uptake observed in water-injected oocytes.

Kinetics

Kinetics were performed in KAAT1 cRNA- and in water-injected oocytes and kinetic parameters of KAAT1-mediated L-leucine uptake were calculated using a multiparameter, iterative, nonlinear regression program (SigmaPlot, Jandel, CA).

L-[^{3}H]leucine concentrations ranged from 25 to 1000 μM (3700–7400 KBq/ml). In Na^+ activation experiments, Na^+ concentrations ranged from 0 to 100 mM and L-[^{3}H]leucine was 0.2 mM.

Results

Ion Selectivity

Leucine uptake measured in KAAT1 expressing oocytes was about tenfold higher compared with control water injected oocytes in the presence of inward Na^+ gradient (Fig. 1). The most peculiar feature of KAAT1-mediated leucine transport is a broad cation selectivity. Accordingly, as shown in Fig. 1, substitution of NaCl with 100 mM LiCl, RbCl or cholineCl allowed a reduced but significant activation of leucine uptakes. The choline effect raises several questions, and particularly whether or not the cotransporter can also work as a uniport, which is to be addressed in the discussion. A modest activation was also observed after substitution of external sodium by 150 mM KCl (insert), although in this condition membrane potential is drastically reduced (*unpublished observations*). The potassium effect, even if significant, cannot be directly compared to those of the other tested cations, but it confirms the unusual ability of this cotransporter to transport K^+ as previously demonstrated with electrophysiological studies (Castagna et al., 1998). To further characterize the ionic dependence of KAAT1-induced transport, we tested the effect of substitution of chloride with other anions. Figure 2 shows that KAAT1-mediated transport is anion dependent since leucine uptake was reduced to 26% of the control by substitution of chloride with gluconate and to 54% by substitution with acetate, whereas thiocyanate had no significant influence on the uptake and presumably it can efficiently substitute for Cl. The chloride dependence observed here has some similarity with that found for GABA transporter GAT2, the only member of GABA family present in peripheral tissues (Borden et al., 1992).

pH Dependence

Since the luminal environment of *Manduca sexta* midgut has a very high pH value, up to 11 (Azuma et al., 1995),

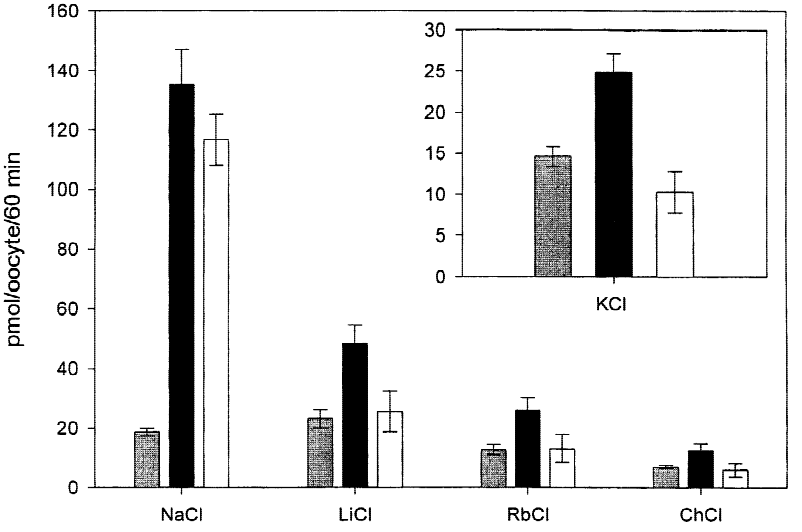

Fig. 1. Uptake of 0.1 mM L-leucine in the presence of 100 mM NaCl, LiCl, RbCl and choline chloride in oocytes injected with water (gray bars) or KAAT1 cRNA (black bars); white bars represent KAAT1-mediated transport. Insert: transport of 0.1 mM L-leucine in the presence of 150 mM KCl. Data are means ± SE of 8–10 oocytes in a representative experiment.

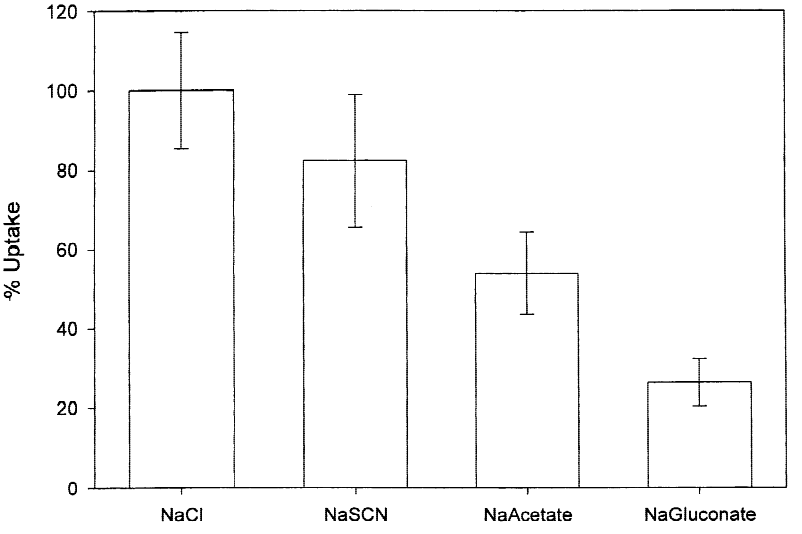

Fig. 2. Anion dependence of 0.1 mM L-leucine uptake. Bars represent KAAT1-induced L-leucine uptake measured in the presence of 100 mM sodium salt of thiocyanate, acetate and gluconate, expressed as a percent of the control performed in the presence of 100 mM NaCl. Data are the means ± SE of three independent experiments performed with 8–10 oocytes.

and several studies have shown that amino acid transport capacity increased at alkaline pH values (Sacchi et al., 1990; Hennigan et al., 1993; Giordana et al., 1998), we tested the pH dependence of leucine uptake in KAAT1 expressing oocytes. KAAT1 dependence on extracellular pH was studied at pH values ranging from 7.9 to 9.8, and the uptake experiments were performed after a short incubation time (5 min) to avoid effects due to oocyte damage. Under these conditions, L-leucine uptake increased about twofold varying the pH values from 7.9 to 9.8 (Fig. 3).

The pH dependence of KAAT1-mediated transport was investigated in terms of the effect on Na^+ activation of L-leucine uptake and leucine kinetics. Figure 4 shows data obtained measuring 0.2 mM L-leucine uptake *vs.* extracellular Na^+ concentration at pH 8.0 and 10.0. Data of induced leucine transport fitted to Michaelis-Menten curves in both the tested conditions. The kinetic parameters were K_{mNa} = 4.6 ± 2 mM, V_{maxNa} = 14.8 ± 1.7 pmol/oocyte/5 min for pH 8.0 and K_{mNa} = 2.8 ± 0.7, V_{maxNa} = 31.3 ± 1.9 pmol/oocyte/5 min for pH 10.0. At the same values of pH, 8.0 and 10.0, L-leucine uptake was also measured as a function of leucine concentration. Figure 5 shows that in both conditions data fitted to a Michaelis-Menten curve with the following parameters: K_m = 120.4 ± 24.2 μM, V_{max} = 23.2 ± 1.4 pmol/oocyte/5 min for pH 8.0 and K_m = 81.3 ± 24.2 μM, V_{max} = 65.6 ± 3.9 pmol/oocyte/5 min for pH 10.0. Kinetic parameters calculated at pH 8 are in good agreement with those determined by electrophysiological studies (Castagna et al., 1998).

Inhibition Experiments

The ability of KAAT1-expressing oocytes to transport amino acids and other organic substrates was tested in a

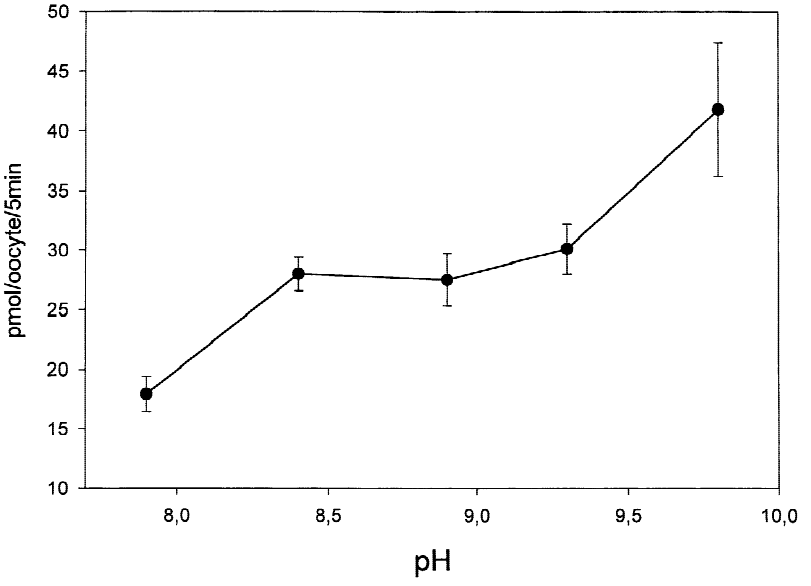

Fig. 3. pH dependence of 0.1 mM L-leucine uptake. Data are the KAAT1-mediated uptake measured at increased values of pH ranging from 7.9 to 9.9 and are means ± SE of 8–10 oocytes in a representative experiment. To avoid oocytes damages at high values of pH, the experiment was performed with an incubation time of 5 min.

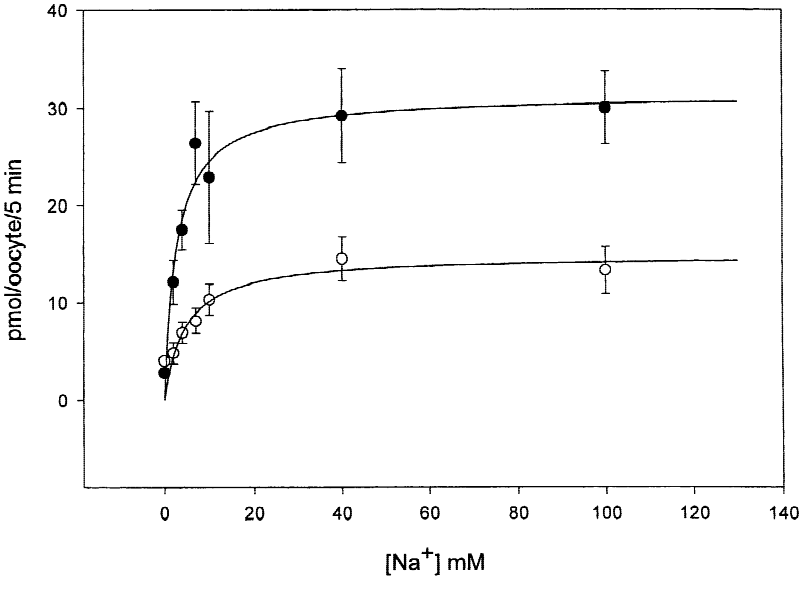

Fig. 4. Na^+ activation of 0.2 mM L-leucine uptake. The curves show KAAT1-mediated leucine uptake measured at different concentrations of external Na^+ at pH 8.0 (white circles) and pH 10.0 (black circles). Values are means ± SE of 8–10 oocytes in a representative experiment and were obtained after an incubation time of 5 min. At concentrations of Na^+ different from 100 mM, osmolarity was maintained with choline.

first set of experiments performed by measuring the uptake of radiolabeled L-leucine, glycine, L-glutamic acid, L-lysine, α-methyl-D-glucopyranoside and D-mannitol, in the presence of 100 mM NaCl at pH 8.0. Figure 6 reports the uptakes of the above compounds in cRNA-injected and water-injected oocytes. In addition to L-leucine, glycine is also recognized as a substrate by KAAT1, whereas acid and basic amino acids (L-glutamic acid, L-lysine), α-methyl-D-glucopyranoside and D-mannitol are not transported. In the subsequent experiments L-leucine was used as a test amino acid and its uptake was always considered to evaluate KAAT1 expression. We also observed that L-leucine uptake in KAAT1-expressing oocytes is linear up to 60 min of incubation (*data not shown*) therefore the experiments were performed measuring leucine uptake in water and in KAAT1 cRNA-injected oocytes after 60 min of incubation.

The substrate selectivity of KAAT1-mediated uptake was then examined by inhibition experiments in which 0.1 mM radiolabeled L-leucine uptake was measured in the presence of 5 mM amino acids or related compounds. The results are expressed as percentages of the control uptakes in the absence of the inhibitors. As Fig. 7 shows, small, bulky, branched and unbranched neutral amino acids exerted a strong inhibition on leucine transport since the uptake was reduced to 4–0% of the control. Amino acids with uncharged polar side chains

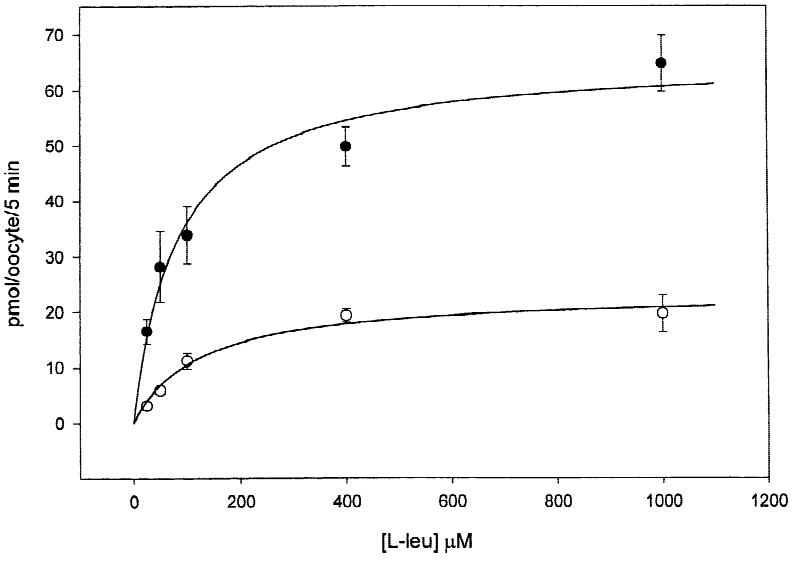

Fig. 5. Kinetics of L-leucine uptake. L-leucine uptake as a function of external leucine concentration was measured in the presence of 100 mM NaCl at pH 8.0 (white circles) and pH 10.0 (black circles). Data are means ± SE of KAAT1-mediated uptake measured for 8–10 oocytes after 5 min of incubation in a representative experiment.

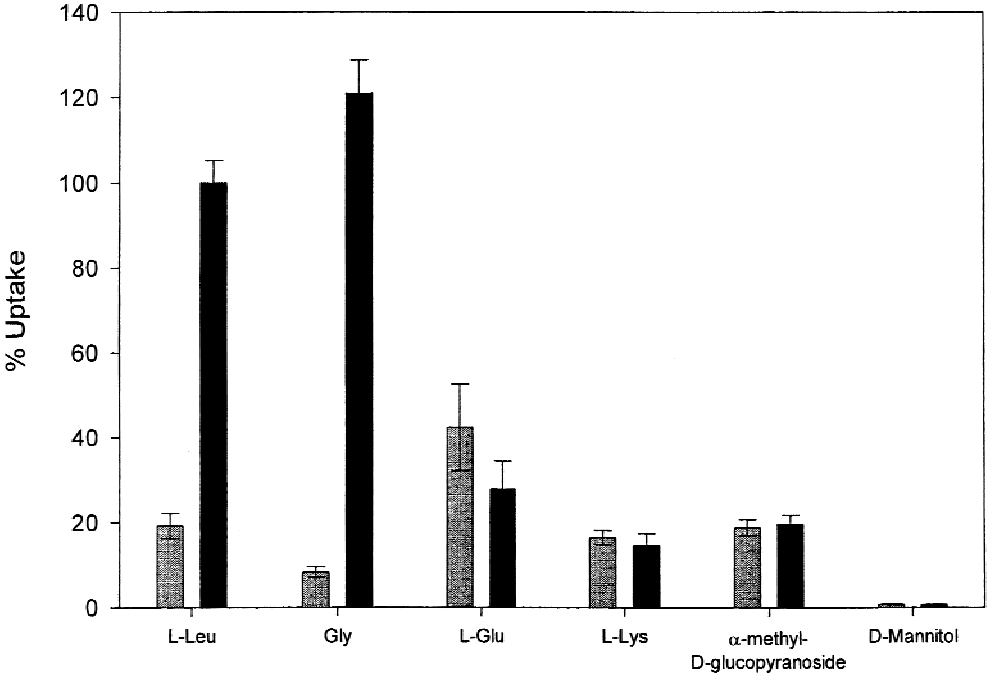

Fig. 6. KAAT1-induced substrate transport. Uptake of 0.1 mM L-leucine, glycine, L-glutamic acid, L-lysine, α-methyl-D-glucopyranoside and D-mannitol in KAAT1 cRNA-injected (black bars) oocytes and water-injected oocytes (gray bars). Data are expressed as percent of the uptake obtained in the presence of L-leucine and values are means ± SE for 8–10 oocytes measured in a representative experiment.

at the tested pH (threonine, serine, glutamine, cysteine and histidine) still have a remarkable inhibitory effect. Data in Fig. 7 show that the presence of those amino acids reduced L-leucine uptake to 10–6% of the control uptake. A similar level of inhibition was observed for glycine that, as shown in the same figure, decreased the uptake to 18% of the control. Proline and hydroxyproline reduced leucine uptake to 30 and 40% respectively (Fig. 7).

The inhibitory effect of amino acids with a charged side chain is also reported in Fig. 7. Among the tested amino acids only lysine showed a significant inhibitory effect (55%), whereas arginine showed a modest inhibition (30%) and glutamate inhibition was not significant. The insert of the figure suggests a relatively low stereospecificity of KAAT1. Both stereoisomers of leucine and methionine exerted a high inhibition of leucine uptakes, but D-enantiomers showed a significantly lower

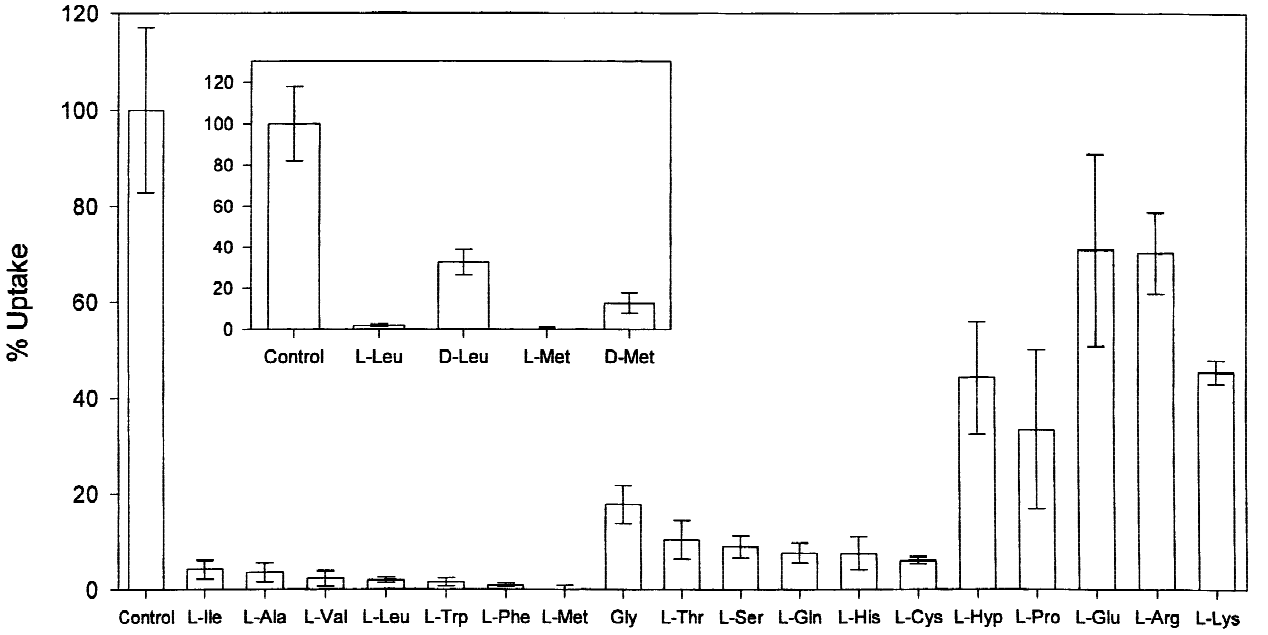

Fig. 7. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM amino acid inhibitor. Data are the KAAT1-mediated transport presented as percent of the control in the absence of inhibitor and values are the means ± SE of three independent experiments performed with 8–10 oocytes.

inhibition than the L-forms. Figure 8 shows the inhibitions exerted by L-phenylalanine (0.2 mM), L-proline (5 mM) and taurine (5 mM) on L-leucine kinetics, the data are presented as Lineweaver-Burk Plot. As expected the tested compounds are competitive inhibitors of leucine uptake.

Due to the strong reduction of L-leucine uptake observed in the presence of unbranched and branched neutral amino acids, we further investigated the influence of side chain modification in terms of lengths or ramification position. Figure 9 shows that the inhibition exerted by unbranched amino acids did not increase with the length of the side chain. L-alanine and homologs, the side chains of which vary between 1 (alanine) and 4 (norleucine) carbon atoms in length, reduced L-leucine uptake to about 5% of the control. Interestingly, the inhibition observed for glycine, an amino acid that lacks a side chain, is lower and significantly different from that of the above compounds. In the insert of Fig. 9 the effects of leucine isomers that differ in the position of the methyl group (isoleucine and norleucine) are compared. All of them were able to inhibit leucine uptake without any significant difference.

The relationship between amino acid structure and inhibition was also evaluated using compounds in which the side chain is characterized by a cyclic structure involving the carbon in α position. As shown in Fig. 10, synthetic amino acids such as BCH (2-amino-2-norbornanecarboxylic acid) and cycloleucine (1-amino-cyclopentane-carboxylic acid), still exerted a strong inhibition of leucine uptake that was reduced to 18 and 3% respectively. In the same figure the effect of methylation of the amino group is also considered. Strong inhibition (90%) was also observed for AIB (α-amino isobutyric acid), whereas no inhibition was detected for its methyl derivative, MeAIB (α-methyl amino isobutyric acid). MeAIB is a model substrate accepted by system A but excluded by system B^0 and apparently also by KAAT1 (Doyle & McGivan, 1992).

The effect of GABA (γ-aminobutyric acid), β-alanine and taurine (2-aminoethanesulfonic acid) on L-leucine uptake was also tested (Fig. 11): GABA and β-alanine caused respectively 10 and 38% inhibition, nevertheless taurine, which possesses a structure similar to β-alanine but with a sulfonic group replacing the carboxylic one, exerted a strong inhibition (72%).

As expected for an amino acid transporter, n-butyric acid and n-butylamine, did not reduce L-leucine uptake. Nor was a β-amino alcohol such as DL-β-hydroxyphenethylamine able to inhibit significantly L-leucine transport (Fig. 12).

Due to pH dependence of KAAT1-mediated transport, we tested the inhibition ability of some amino acids at pH 5.5, 8.0 and 10.0. We selected representative molecules of the three classes of amino acids: neutral (L-phenylalanine); acid (L-glutamic acid) and basic (L-lysine). Figure 13 shows that the inhibitory effect of L-

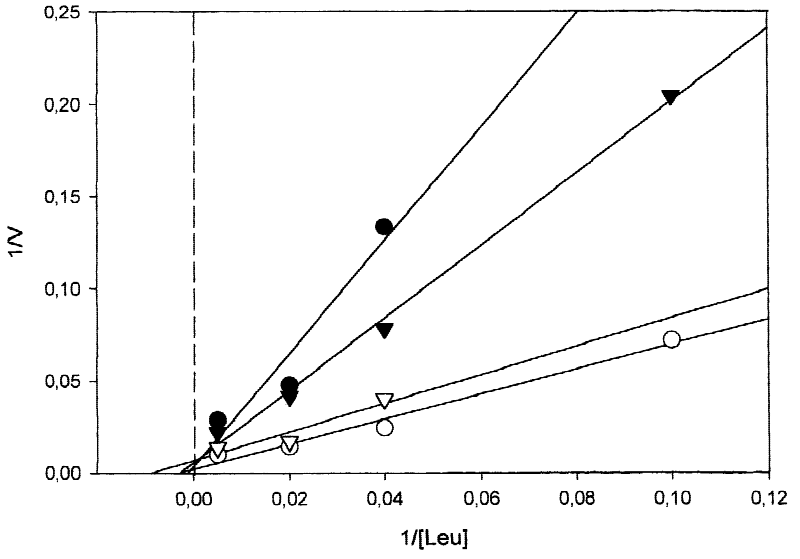

Fig. 8. Inhibition kinetics of L-leucine uptake. L-leucine uptake as a function of external leucine concentration was measured in the absence (white circles) or in the presence of taurine 5 mM (white triangles), L-phenylalanine 0.2 mM (black triangles), L-proline 5 mM (black circles). Data are the KAAT1-mediated transport in a representative experiment performed with 8–10 oocytes.

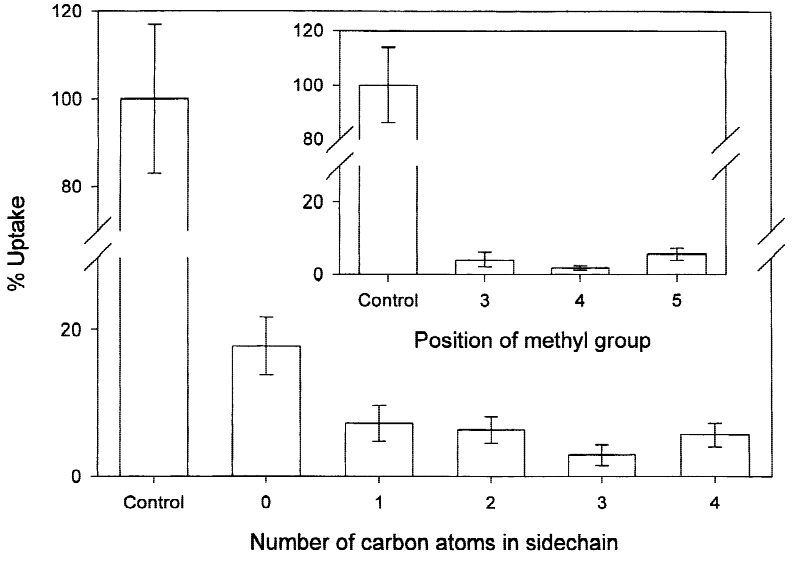

Fig. 9. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM of L-alanine homologues: glycine, L-alanine, L-α-amino-n-butyric acid, L-norvaline and L-norleucine. Insert: inhibition exerted by L-isoleucine, L-leucine and L-norleucine. Data are the KAAT1-mediated transport presented as percent of the control in the absence of inhibitor and values are the means ± SE of three independent experiments performed with 8–10 oocytes.

phenylalanine was not influenced by variation of extracellular pH, whereas L-glutamic acid was not able to inhibit L-leucine uptake at pH 8.0 but showed 100% inhibition at pH 5.5 and 58% inhibition at pH 10.0 compared to control uptake at the same pH. L-lysine inhibition of L-leucine uptake was 41% at pH 8, 24% at pH 5.5 and 87% at pH 10. The insert of the figure shows that control L-leucine uptakes in the absence of inhibitors increased sixfold varying the pH value from 5.5 to 10.0.

Discussion

The expression of a transporter in *Xenopus laevis* oocytes greatly simplifies the study of the physiological features of a protein and allows attributing some properties to a specific molecule rather than to a population of similar but not identical transporters. This is particularly true for amino acid transporters that often present overlapping selectivity for substrates and differ in only a few aspects. The number of cloned amino acid transporters is rapidly increasing and they can usually be grouped in families (Palacin et al., 1998). The amino acid sequence of KAAT1 indicates a modest but significant identity with the superfamily of GABA transporters. The structures of proteins belonging to this family are predicted to have 12 membrane-spanning domains and a large extracellular loop between helices 3 and 4. These features are also shared by KAAT1. In addition, these transporters have a channel mode of ion conduction in the absence of

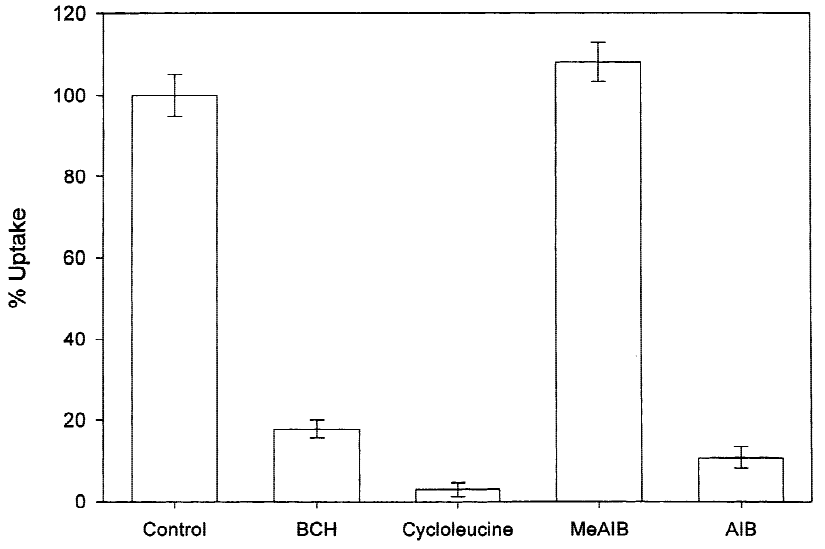

Fig. 10. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM 2-amino-2-norbornane carboxylic acid (BCH), cycloleucine, α-N-methylamino-isobutyric acid (MeAIB) and α-amino-isobutyric acid (AIB). Data are the KAAT1-mediated transport presented as percent of the control in the absence of inhibitor and values are the means ± SE of three independent experiments performed with 8–10 oocytes.

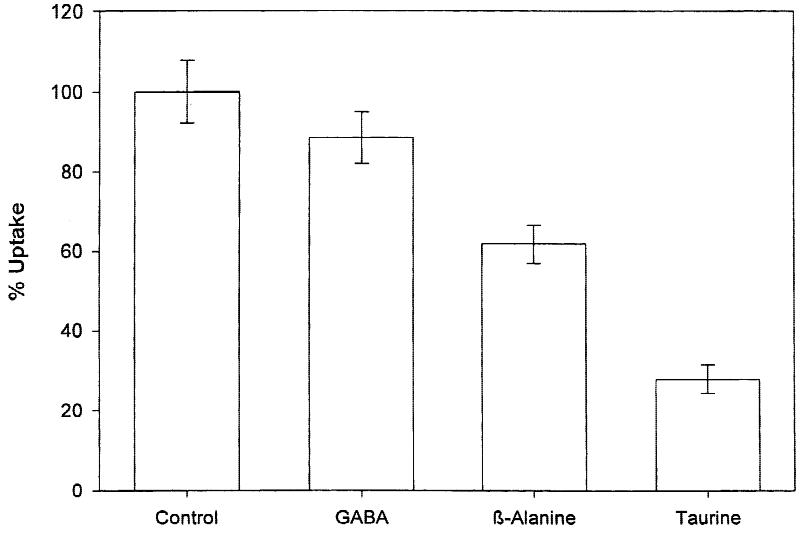

Fig. 11. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM γ-amino-butyric acid (GABA), β-alanine and taurine. Data are the KAAT1-mediated transport presented as percent of the control in the absence of inhibitor and values are the means ± SE of three independent experiments performed with 8–10 oocytes.

the organic substrates as shown by the uncoupled currents measured in electrophysiological experiments (Mager et al., 1993; Bossi et al., 1999). The GABA transporters are Na^+ and Cl^- coupled but GAT-1, GAT-2, and GAT-3 vary in their dependence on external Cl^-, and KAAT1 seems to be more similar to GAT-2 since when Cl^- was substituted by acetate, transport by GAT-2 was decreased to 43% of the control and leucine transport by KAAT1 was decreased to 54% (Fig. 2) (Borden, 1996). Furthermore, the chloride-dependence is not strict since other pseudohalide anions such as thyocyanate are accepted. Cation dependence shows a clear distinction between GABA transporters and KAAT1 since the latter exhibits broader cation selectivity accepting K^+ and Li^+ (Fig. 1). This figure also shows a significant mean value of leucine transport in the absence of Na^+, however this result was not observed in all the experiments. In this kind of experiment Na^+ was substituted by choline but the hypothesis that this cation can drive L-leucine uptake is ruled out by the experiments showing that choline does not cause any inward current in the presence of L-leucine (Castagna et al., 1998). Alternatively, the transporter could operate either as a cotransporter or as a uniporter. This hypothesis is very attractive and is also supported by some previous results obtained in brush border membrane vesicles (Sacchi et al., 1990; Giordana et al., 1998; Leonardi et al., 1998), however, as shown in Fig. 4, the K_{mNa} is 2–4 mM, and therefore a small amount of Na^+ extruded by the Na^+/K^+-ATPase might easily activate the transporter expressed in oocyte. Accordingly, preliminary experiments performed in the absence of sodium and in the presence of ouabain showed no KAAT1-mediated L-leucine uptake (*data not shown*). The high affinity of KAAT1 for Na^+

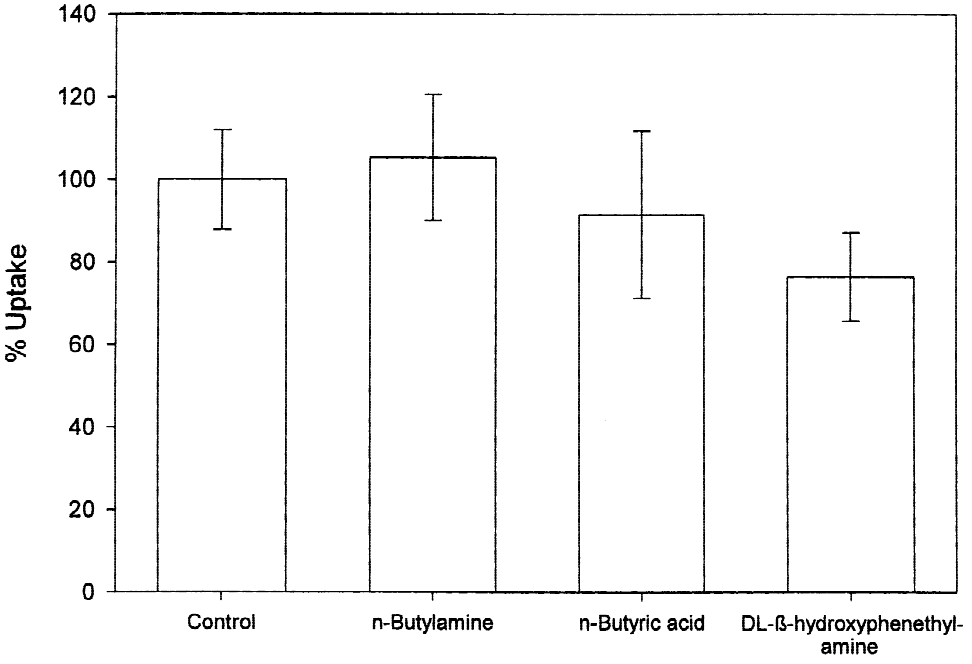

Fig. 12. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM n-butylamine, n-butyric acid and DL-β-hydroxyphenethyl-amine. Data are the KAAT1-mediated transport presented as percent of the control in the absence of inhibitor and values are the means ± SE of several experiments performed with 8–10 oocytes.

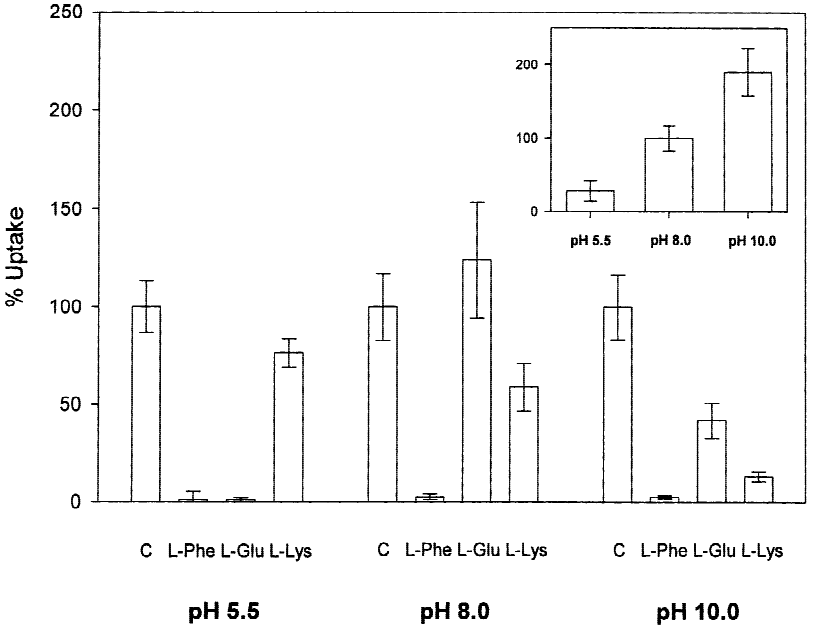

Fig. 13. Inhibition of KAAT1 induced L-leucine uptake. Uptake of 0.1 mM L-leucine measured in the presence of 5 mM L-phenylalanine, L-glutamic acid and L-lysine at pH 5.5, 8.0 and 10.0. Bars present KAAT1-mediated transport as percent of the corresponding control in the absence of inhibitor. Insert: uptake of 0.1 mM L-leucine at pH 5.5, 8.0 and 10.0, expressed as percent of the control at pH 8. Data are the means ± SE of three independent experiments performed with 8–10 oocytes. Experiments were performed at 5 min of incubation.

is a further difference with GABA transporters that have an affinity for Na^+ one order of magnitude lower (Mager, 1996). Compared with other amino acid transporters, KAAT1 presents a broadening of the cation specificity and an increased Na^+ affinity which are probably related to the diet of these larvae. Indeed, the absorption of an essential ion such as Na^+ is a major problem for lepidopteran larvae that have a diet with high potassium and low sodium content. Therefore, the kinetic features of KAAT1 may have the function of ensuring sodium and amino acid uptake in an epithelium which contains no conventional sodium pump (Sacchi et al., 1999).

Many studies in different species have shown that the amino acid transport in membrane vesicles from lepi-

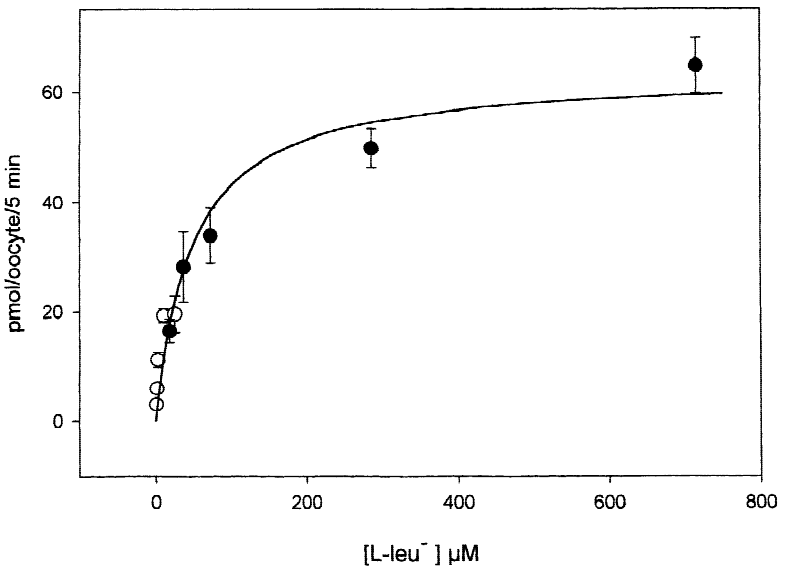

Fig. 14. Elaboration of L-leucine kinetics as a function of the concentration of anionic form of leucine. White circles represent data of kinetics performed at pH 8.0 and black circles data of kinetics performed at pH 10.0. The concentration of anionic form of the amino acid was calculated using Handerson-Hasselbach equation.

dopteran larvae increased under high pH conditions resembling the in vivo conditions of the midgut (Sacchi et al., 1990; Hennigan et al., 1993; Giordana et al., 1998).

The results reported here show a large increase of KAAT1 mediated L-leucine uptake at high pH values (Fig. 3). This uptake increase could be due to pH effects on the transport protein and/or on substrate charges, since the α-amino group is deprotonated in alkaline solutions and neutral amino acids bear a net negative charge. It should be noted that the steepest uptake increase is observed near the pH value corresponding to L-leucine pK_2 (9.7). A H^+- or OH^--coupled KAAT1 mediated amino acid transport seems to be excluded on the basis of experiments performed with membrane vesicles, although a pH gradient can energize transport processes (Sacchi et al., 1990; Giordana et al., 1998). Besides, an inward directed proton gradient (pH_{out} = 5.5) causes a reduction of L-leucine uptake (Fig. 13), which seems to indicate that, despite the broad cation specificity of KAAT1, H^+ cannot act as a driver for amino acid uptake. KAAT1 transport activity is favoured by the high extracellular pH that causes an increase of leucine V_{max} whereas the K_m values for both leucine and Na^+ are not affected. However, if leucine kinetics are plotted as a function of the anionic form of leucine (Fig. 14) a single hyperbolic curve results and at different pH values, similar uptakes are obtained for similar anionic leucine concentrations. This result supports the hypothesis that the pH effect is mainly on the amino acid rather than on the transport protein.

The lumen alkalinization in the midgut of lepidopteran larvae, probably involved in the control of bacterial infections, in the reduction of leaf toxicity, and in the increase of protein solubility, also exerts an influence on amino acid absorption mediated by KAAT1.

The high inhibition of L-leucine uptake observed in the presence of glycine (Fig. 7) and the high KAAT1-mediated uptake of radiolabeled glycine (Fig. 6) indicate that the presence of a side chain is not a critical parameter for the substrate structure. The presence of a neutral apolar side chain on the α-amino acidic structure raises slightly the inhibition (Fig. 7), but modifications of the side chain in terms of size, length, presence and position of ramification do not influence inhibition of L-leucine transport (Fig. 9) as also observed in brush border membrane vesicles (Parthasarathy et al., 1994). Charged groups on the side chain present in ionic amino acids such as L-glutamic acid, L-lysine and L-arginine, have a destabilizing effect on the interaction inhibitor-transporter (Fig. 7), moreover L-glutamic acid and L-lysine, as shown in Fig. 6 are not transported by KAAT1 at pH 8. Polar groups that, at the tested pH are uncharged (imidazole in histidine, hydroxyl in serine and threonine) or only partially charged (thiol in cysteine) do not significantly decrease the inhibition ability of the molecule (Fig. 7). Nevertheless, as already shown for cloned transporters, ATB^0 and ASCT1 and ASCT2 (Kekuda et al., 1997; Tamarappoo et al., 1996; Tate et al., 1996), L-glutamic acid is able to inhibit KAAT1 mediated L-leucine uptake at acid pH (Fig. 13). These data are probably related to the increase of glutamic acid with a non-ionized side chain. In fact, the variation of pH has no effect on inhibition exerted by L-phenylalanine which lacks ionizable groups on the side chain (Fig. 13). Interestingly an increased inhibitory effect is observed for L-glutamic acid also at pH 10 (Fig. 13), possibly due to deprotonation of the α-amino group. L-lysine inhibition of L-leucine uptake increases at increasing pH values, and it is highest at pH 10 when the side chain is only partially charged and the α-amino group is deprotonated.

A cyclic side chain involving the carbon in position α, as in the synthetic α-amino acids cycloleucine and in the more complex BCH, still allows a good inhibitory effect (⩾80%) (Fig. 10). The lack of L-leucine uptake inhibition by n-butyric acid, n-butylamine and β-hydroxyphenethylamine indicates that the presence of both functional groups of the amino acid molecule is strictly necessary for an inhibitory compound (Fig. 12). In particular the amino group must be located in α position, indeed a drastic reduction of the inhibitory ability was observed for β-aminopropionic acid (β-alanine), whereas no inhibition was observed for γ-aminobutyric acid (GABA) (Fig. 11) despite the similarity of KAAT1 with GABA transporters. An exception to the required distance between the functional groups is the relatively high inhibition exerted by the sulphur β-amino acid derivative, taurine (Fig. 11). Methylation of α-amino group is also a negative characteristic in terms of inhibition, since L-leucine uptake was drastically reduced by AIB (α-amino-isobutyric acid) but not by its N-methyl derivative MeAIB (α-methylamino-isobutyric acid) (Fig. 10). Besides, the requirement of a unsubstituted α-amino group was already suggested by the reduced inhibitory effect of the imino acid proline (Fig. 7).

The effects of the D-enantiomers on the KAAT1 induced L-leucine uptake reveal a small chiral discrimination of binding interactions between the cotransporter and the substrate since a significant inhibition is observed for D-leucine and D-methionine, but the effects are significantly lower compared with the corresponding enantiomeric forms (Fig. 7 insert).

In spite of the structural and ionic dependence similarities with several members of the GABA transporters' family, the inhibitions observed for neutral branched and unbranched amino acids, the weak inhibition exerted by ionic amino acids and the lack of effect of GABA and MeAIB, indicate that KAAT1 has a substrate selectivity similar to system B^0. This transport system has been described in mammal intestinal and renal epithelium (Stevens et al., 1982; Lynch et al., 1987) and is involved in the absorption and reabsorption of neutral amino acids. It is worth mentioning the high inhibition of anionic amino acids at low pH observed for the cloned transporter ATB^0 belonging to transport system B^0 (Kekuda et al., 1997) and also observed for KAAT1 mediated transport (Fig. 13). The same pH dependence of interaction between an acid amino acid and transporter has also been described for ASCT1 and ASCT2 (Tamarappoo et al., 1996; Tate et al., 1996), transporters belonging to system ASC that unlike KAAT1, transport only small aliphatic amino acids (Christensen et al., 1967).

In conclusion, the structural requirements for a KAAT1 organic substrate can be summarized as follows: (i) a carboxylic group, (ii) an unsubstituted α-amino group, (iii) the side chain is unnecessary, if present it should be uncharged regardless of length and ramification.

KAAT1 can transport L-leucine at pH 5.5 and 8, but the maximum uptake has been observed at pH 10 when amino and carboxylic groups are both deprotonated, which suggests that a deprotonated α-amino group is a positive feature for a KAAT1 substrate.

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References

Azuma, M., Harvey, W.R., Vieczorek, H. 1995. Stoichiometry of K^+/H^+ antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett.* **361:**153–156

Borden, L.A., Smith, K.E., Hartig, P.R., Branchek, T.A., Weinshank, R.L. 1992. Molecular heterogeneity of the γ-aminobutyric acid (GABA) transporter system. *J. Biol. Chem.* **267:**21098–21104

Borden, L.A. 1996. GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem. Int.* **29:**335–356

Bossi, E., Centinaio, E., Castagna, M., Giovannardi, S., Vincenti, S., Sacchi, V.F., Peres, A. 1999. Ion permeation and binding at the lepidopteran amino acid transporter KAAT1 expressed in *Xenopus* oocytes. *J. of Physiol.* **513:**729–742

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R., Hediger, M.A. 1997. Molecular characteristics of mammalian and insect amino acid transporters: implications for amino acid homeostasis. *J. Exp. Biol.* **200:**269–286

Castagna, M., Shayakul, C., Trotti, D., Sacchi, V.F., Harvey, W.R., Hediger, M.A. 1998. Cloning and characterization of a potassium-coupled amino acid transporter. *Proc. Natl. Acad. Sci. USA* **95:**5395–5400

Christensen, H.N., Liang, M., Archer, E.G. 1967. A distinct Na^+-requiring transport system for alanine, serine, cysteine, and similar amino acids. *J. Biol. Chem.* **242:**5237–5246

Doyle, F.A., McGivan, J.D. 1992. The bovine renal epithelial cell line NBL-1 expresses a broad specificity, Na^+-dependent neutral amino acid transport system (System B^0) similar to that in bovine renal brush border membrane vesicles. *Biochim. Biophys. Acta* **1104:**55–62

Dow, J.A.T. 1992. pH gradients in lepidopteran midgut. *J. Exp. Biol.* **172:**355–375

Giordana, B., Sacchi, V.F., Hanozet, G.M. 1982. Intestinal amino acid absorption in lepidopteran larvae. *Biochim. Biophys. Acta* **692:**81–88

Giordana, B., Leonardi, M.G., Casartelli, P., Consonni, P., Parenti, P. 1998. K^+-neutral amino acid symport of *Bombix mori* larval midgut: a system operative in extreme conditions. *Am. J. Physiol.* **274:**R1361–R1371

Harvey, W.R., Wieczorek, H. 1997. Energization of animal plasma membranes by chemiosmotic H^+ V-ATPases. *J. Exp. Biol.* **200:** 203–216

Harvey, W.R., Maddrell, S.H.P., Telfer, W.H., Wieczorek, H. 1998. H^+ V-ATPases energizes animal plasma membranes for secretion and absorption of ions and fluids. *Am. Zoologist* **38:**426–441

Hennigan, B.B., Wolfersberger, M.G., Parthasarathy, R., Harvey, W.R. 1993. Cation-dependent leucine, alanine, and phenylalanine uptake at pH 10 in brush-border membrane vesicles from larval *Manduca sexta* midgut. *Biochim. Biophys. Acta* **1148:**209–215

Kekuda, R., Torres-Zamorano, V., Fei, Y.J., Prasad, P.D., Li, H.W., Mader, L.D., Leibach, F.H., Ganapathy, V. 1997. Molecular and functional characterization of intestinal Na^+-dependent neutral amino acid transporter B^0. *Am. J. Physiol.* **272:**G1463–G1472

Leonardi, M.G., Casartelli, P., Parenti, P., Giordana, B. 1998. Evidence

for a low affinity, high-capacity uniport for amino acids in *Bombix mori* larval midgut. *Am. J. Physiol.* **274:**1372–1375

Lynch, A.M., McGivan, J.D. 1987. Evidence for a single common Na^+-dependent transport system for alanine, glutamine, leucine and phenylalanine in brush border membrane vesicles from bovine kidney. *Biochim. Biophys. Acta* **899:**176–184

Mager, S., Naeve, J., Quick, M., Labarca, C., Davidson, N., Lester, H.A. 1993. Steady states, charge movements, and rates for a cloned GABA transporter expressed in *Xenopus* oocytes. *Neuron* **10:**177–188

Mager, S., Kleinberger-Doron, N., Keshet, G.I., Davidson, N., Kanner, B.I., Lester, H.A. 1996. Ion binding and permeation at the GABA transporter GAT1. *J. of Neurosc.* **16:**5405–5414

Palacin, M., Estevez, R., Bertran, J., Zorzano, A. 1998. Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol. Rev.* **78:**969–1054

Parthasarathy, R., Xie, T., Wolfersberger, M.G., Harvey, W.R. 1994. Substrate structure and amino acid K^+-symport in brush border membrane vesicles from larval *Manduca sexta* midgut. *J. Exp. Biol.* **197:**237–250

Sacchi, V.F., Giordana, B., Campanini, F., Bonfanti, P., Hanozet, G.M. 1990. Leucine uptake in brush border membrane vesicles from the midgut of a lepidopteran larva, *Philosamia cynthia. J. Exp. Biol.* **149:**207–221

Sacchi, V.F., Castagna, M., Trotti, D., Shayakul, C., Hediger, M.A. 1999. Neutral amino acid absorption in the midgut of lepidopteran larvae. *J. Insect. Physiol.* (*in press*)

Stevens, B.R., Ross, H.J., Wright, E.M. 1982. Multiple transport pathways for neutral amino acids in jejunal brush border vesicles. *J. Membrane Biol.* **66:**213–225

Tamarappo, B.K., McDonald, K.K., Kilberg, M.S. 1996. Expressed human hippocampal ASCT1 amino acid transporter exhibits a pH-dependent change in substrate specificity. *Biochim. Biophys. Acta* **1279:**131–136

Tate, N.U., Endou, H., Kanai, Y. 1996. Cloning and functional characterization of a system ASC-like Na^+-dependent neutral amino acid transporter. *J. Biol. Chem.* **271:**14883–14890

Wieczorek, H., Weerth, S., Schindlbeck, M., Klein, U. 1989. A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264:**11143–11148