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How many Na$^+$-dependent carriers for L-alanine and L-proline in the eel intestine? Studies with brush-border membrane vesicles

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Using brush-border membrane (BBM) vesicles prepared from the intestine of the European eel, the specificity of L-alanine and L-proline Na$^+$-dependent transport was investigated by measuring the uptake of isotopically labelled substrates. In the presence of Na$^+$ ions, cross-inhibition between alanine and proline transports was observed; in addition α-(methylamino)isobutyric acid (MeAIB) inhibited proline but had no effect on alanine uptake. These results can be explained by the presence, in eel intestinal BBM vesicles, of at least two distinct agencies for Na$^+$-dependent proline and alanine translocation. The first system is specific for alanine and short-chain neutral amino acids; the second system, specific for imino acids and the N-methylated analogues, is regulated by alanine concentration.

Introduction

Amino-acid transport systems present on plasma membrane have been extensively studied in a number of different cells and tissues, from bacteria to mammals [1–4].

With respect to neutral amino acids, using a variety of nonepithelial cell-types, Christensen [5] has suggested that Na$^+$-dependent transport of neutral L-amino acids takes place via: (a) A (alanine preferring) system; (b) ASC (alanine, serine, cysteine preferring) system; (c) Gly (glycine preferring) system; moreover: (a) N-methylated amino acids as α-(methylamino)isobutyric acid (MeAIB) can be transported only by system A: (b) A and Gly systems have never been observed in the same plasma membrane.

On the other hand, studies with rabbit intestinal isolated plasma membranes (brush-border and basolateral) led Wright's group to propose a different classification [6]. According to these authors, neutral amino-acid Na$^+$-dependent transports occur via: (a) NBB (neutral brush-border) system; (b) IMINO (imino acids preferring) system; (c) PHE (phenylalanine preferring) system. The NBB agency is in charge for small neutral amino acids, while larger amino acids are transported by the PHE system; the IMINO system physiologically transports prolines and is specific for N-methylated amino acids.

Recently we have succeeded in isolating BBM vesicles from the intestine of the eel [7], a carnivorous and euryhaline teleost [8]. Using this preparation, after the identification and characterization of the pathways for alanine and proline [7,10], we approached the intriguing problem of the overlapping specificity shown by the two Na$^+$-dependent transport systems. To shed more light on the general topic of the neutral amino-acid translocation, this paper faces the problem of the identification of the number of agencies responsible for alanine and proline Na$^+$-dependent transport, present on the intestinal brush-border membrane of the eel. In this paper we have investigated the inhibition of the following Na$^+$-dependent uptakes: (1) proline uptake by alanine and MeAIB; (2) alanine uptake by proline and MeAIB; (3) MeAIB by proline and alanine.

Materials and Methods

Materials. European yellow eels (*Anguilla anguilla*) (150–200 g) were obtained from Ittica Ugento (Lecce, Italy) and kept in sea-water aquariums. All chemicals, reagent grade, were purchased from Merck (Darmstadt, F.R.G.); valinomycin was obtained from Sigma (St.

Abbreviations: BBM, brush-border membrane; MeAIB, α-(methylamino)isobutyric acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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Louis, MO). Isotopically labelled amino acids were obtained from Du-Pont New England Nuclear (Boston, MA). All amino acids were L-stereoisomeric.

Preparation of BBM vesicles. BBM vesicles were prepared from the intestine of yellow eels, as described elsewhere [7]: the preparation is based on selective precipitation, in the presence of EGTA and $MgCl_2$ 12 mM, of all cellular components, with the exception of BBM. The BBM fraction routinely showed enrichment factors over the initial homogenate of at least 16 for leucine aminopeptidase, 12 for maltase and 13 for alkaline phosphatase [11–13]; no enrichment was found for marker enzymes of mitochondria, endoplasmic reticulum and basolateral membrane [7]. After the last centrifugation step, BBM vesicles were resuspended in the buffer chosen for the experiment, centrifuged again at 50000 × *g* for 30 min, and finally resuspended by passing them 30-times through a fine gauge needle. Details of buffer composition are given in the legends to the figures. Protein concentration was measured with the Bio-Rad kit (using lyophilized bovine plasma γ-globulin as standard) and adjusted to the indicated concentration.

Uptake measurement. The uptake of L-[^{3}H]alanine, L-[^{3}H]proline and [^{14}C]MeAIB was measured at 25°C, by a rapid filtration technique, using a rapid semiautomatic measurement apparatus (Innovativ Labor A.G., Adliswill, Switzerland). With this instrument, uptake was started by adding 10 μl (100 μg protein) of vesicle suspension of 40 μl of incubation medium; after the indicated time, the reaction was automatically stopped by injecting 3 ml of ice-cold stop solution into the reaction tube. The mixture of 3 ml stop solution plus vesicles was rapidly filtered through a Millipore filter (HAWP pore size = 0.45 μm) kept under suction. The filter was washed with 3 ml of stop solution and assayed for radioactivity (Beckman LS 1800 and Ready Solv EP scintillation fluid). The nonspecific binding of the isotopes to filters was determined by measuring the uptake values at zero time. The stop solution composition was (in mM) 50 KCl, 20 Hepes adjusted to pH 7.4 with Tris and NaCl up to the same total osmolarity of the incubation medium.

The experimental conditions were invariable: (a) zero-trans conditions (absence of amino acid and Na^+-ions in the intra-vesicular medium); (b) short-circuited membrane potential. Changes in membrane potential were prevented by using in the intra- and extra-vesicular media, 50 mM KCl and 12.5 μg/mg protein valinomycin (added as a stock solution in ethanol, leading to 21.4 mM ethanol final concentration).

Presenting data and statistics. Each experimental condition was tested on at least two different membrane preparations. The figures report representative experiments; the points shown in figures are averages (±S.E.) of at least four replicates. To fit equations of competitive and noncompetitive inhibition [14] to our data, the nonlinear regression subroutine of the program Statgraphics (STSC, Rockville, MD, U.S.A.) and an M28 personal computer (Olivetti, Ivrea, Italy) were used. The nonlinear regression procedure calculates least-squares estimates of the parameters; the algorithm used was developed by Marquardt [15]. Information about fitting curves to data using nonlinear regression can be found elsewhere [16].

Results and Discussion

Inhibition of Na$^+$-dependent alanine uptake by proline and MeAIB

In BBM vesicles from the eel intestine, a Na-dependent alanine pathway, (K_{app} = 1.29 ± 0.14 mM, J_{max} = 3.61 ± 0.17 nmol/mg protein per min)) has been already demonstrated [7]. The inhibition by proline and glucose (as control) of alanine (0.6 mM) uptake was studied (Fig. 1) in the presence and absence of Na^+ ions in the extravesicular medium. In the presence of Na^+ ions (closed symbols), both glucose (triangles) and proline (circles) inhibited the alanine uptake; moreover, a 10 mM inhibitor concentration reduced the alanine uptake at the values measured in the absence of Na^+ ions (open symbols). These results confirm the suggestion [7] that L-alanine uptake, in BBM vesicles from the eel intestine, takes place via at least three pathways according to the following equation:

$$J_{Ala} = J_{Na} + J_{chol} + (P \cdot [Ala])$$

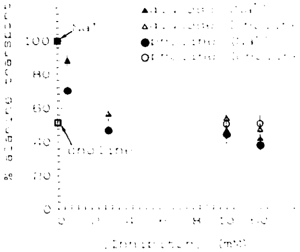

Fig. 1. Inhibition by L-proline and glucose of L-alanine (4 s) uptake in BBM vesicles from the eel intestine. Vesicles, prepared in a buffer containing (in mM) 375 mannitol, 50 KCl, 20 Hepes adjusted to pH 7.4 with Tris, were incubated in a medium containing (final concentration in mM) 175 mannitol, 50 KCl, 0.0224 valinomycin, 21.4 ethanol, 0.6 L-alanine, 20 Hepes adjusted to pH 7.4 with 100 Tris-NaCl (closed symbols) or choline chloride (open symbols), and L-proline (circles) or glucose (triangles) ranging from 0 to 50. The control uptake values (0 mM inhibitor) were 5.03 ± 1.07 and 2.60 ± 0.23 nmol/mg protein per min, respectively in the presence and absence of Na^+ ions.

TABLE I

Inhibition of Na^+-dependent alanine (5 s) and Na^+-dependent proline (4 s) uptake in BBM vesicles from the eel intestine

The alanine and proline data were obtained from different membrane preparations. The experimental conditions were similar to those of Fig. 2. The control uptake values (100%) were (in nmol/mg protein per min) 1.18 ± 0.32 for alanine and 1.38 ± 0.15 for proline.

Substrate	Inhibitor		Transport
Alanine (0.9 mM)	none		100
	MeAIB	2 mM	94 ± 25
	MeAIB	5 mM	101 ± 20
Proline (0.3 mM)	none		100
	alanine	3 mM	79 ± 8
	alanine	10 mM	60 ± 10
	MeAIB	3 mM	40 ± 8
	MeAIB	10 mM	15 ± 5

where J_{Na} and J_{chol} indicate two saturable components whose dependence on substrate concentration is described by the Michaelis-Menten equation, $J = (J_{max} \cdot [Ala])/(K_{app} + [Ala])$. The most surprising result of Fig. 1 was the glucose inhibition of Na^+-dependent alanine uptake. Previous papers from our laboratory reported that: (1) J_{max} values for Na^+-dependent glucose and Na^+-dependent proline were comparable (6.87 ± 0.84 and 5.68 ± 1.78 nmol/mg protein per min, respectively [7,10]); (2) n = Hill index [14] resulted as 2 for Na^+/glucose cotransport [9] and 1 for Na^+/proline cotransport [10]. We therefore think that glucose inhibited the Na^+-dependent alanine uptake (Fig. 1) by collapsing the Na^+ gradient, while 0.4 mM proline inhibited Na^+-dependent alanine uptake by a more specific mechanism. The kinetics of inhibition by proline of Na^+-dependent alanine uptake was studied in more detail. Unfortunately, in this experiment (which was repeated 3 times), inhibition was clearly observed, but the data obtained in the presence of different concentrations of inhibitor were not statistically different,in spite of precautions taken; these negative results arose from the difficulty to measure the Na^+-dependent alanine uptake (obtained as difference between values measured in the presence and absence of Na^+ ions).

The first part of Table I reports the lack of inhibition by α-(methylamino)isobutyric acid (MeAIB) on alanine uptake; the same result was obtained using an alanine concentration from 0.45 up to 2.6 (mM) and an MeAIB concentration from 0.1 up to 5 (data not shown).

In brief, the Na^+-dependent alanine uptake was: (1) completely inhibited by proline but it was not possible to establish the type of inhibition; (2) not inhibited at all by MeAIB.

Inhibition of Na^+-dependent proline by MeAIB and alanine

It has already been shown [10] that proline uptake, in BBM vesicles of the eel intestine, occurs according to the following equation:

$$J = J_{Na} + (P \cdot [Pro])$$

where J_{Na} indicates a flux component with a Michaelian behavior (see above). The inhibition of the Na^+-dependent proline uptake (4 s) by alanine and MeAIB was studied; the preliminary results reported in the second part of Table I, indicate that MeAIB when tested as an inhibitor of proline uptake, was more effective than alanine.

The inhibition by MeAIB (Fig. 2) and by alanine (Fig. 3) of Na^+-dependent proline uptake was then investigated in detail; the results are reported according to the Woolf-Augustinsson-Hofstee linear transformation of the Michaelis-Menten equation [14]. Visual inspection of Fig. 2 (MeAIB inhibition of Na^+-dependent proline uptake) suggests that the lines interpolating the rates at any inhibitor concentration, have a common intercept on the Y-axis consistent with competitive inhibition. The following equation [14]:

$$J = \frac{J_{max} \cdot [Pro]}{K_{app} \cdot (1 + ([MeAIB]/K_i)) + [Pro]}$$

was fitted to our data with a nonlinear regression subroutine (see Materials and Methods). Over two experiments, the following values for kinetic parameters were obtained: J_{max} (nmol/mg protein per min) = 8.18 ± 0.78

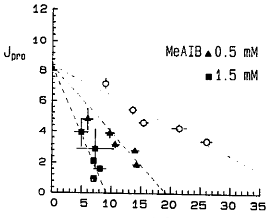

Fig. 2. Inhibition by MeAIB of Na-dependent L-proline uptake (4 s) in BBM vesicles from the eel intestine. Units are J_{pro} = nmol/mg protein per min, [proline] = mM. Vesicles, prepared in a buffer containing (in mM) 300 mannitol, 50 KCl, 20 Hepes adjusted to pH 7.4 with Tris, were incubated in a medium containing (final concentration in mM) 100 mannitol, 100 NaCl, 50 KCl, 0.0224 valinomycin, 1.4 ethanol, 20 Hepes adjusted to pH 7.4 with Tris, proline ranging from 0.133 to 0.8, and 0 MeAIB (circles) or 0.5 (triangles) or 1.5 (squares). Plotted values were obtained by subtraction from data obtained in the presence of Na^+ ions, those measured in the presence of choline ions (data not shown). The equation of competitive inhibition was fitted to data with a nonlinear regression program; kinetic parameters resulted as J_{max} (nmol/mg protein per min)) = 8.18 ± 0.78; K_{app} (mM) = 0.19 ± 0.05; K_i (mM) = 0.41 ± 0.09; the dashed lines represent the best curves fitting the data.

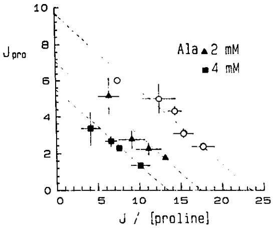

Fig. 3. Inhibition by L-alanine of Na$^+$-dependent L-proline (4 s) uptake in BBM vesicles from the eel intestine. Experimental conditions were similar to those of Fig. 2; alanine concentration in the incubation medium was (in mM) 0 (circles) or 2 (triangles) or 4 (squares). The equation of noncompetitive inhibition was fitted to data with a nonlinear regression program; kinetic parameters were determined to be J_{max} (nmol/min per mg protein) = 9.65 ± 1.63; K_{app} (mM) = 0.41 ± 0.14; K_i (mM) = 5.42 ± 1.04; the dashed lines represent the best curves fitting the data.

and 5.83 ± 0.59; K_{app} (mM) = 0.19 ± 0.05 and 0.12 ± 0.04; K_i (mM) = 0.41 ± 0.09 and 0.34 ± 0.10.

On the other hand, visual inspection of Fig. 3 (inhibition by alanine of Na$^+$-dependent proline uptake) suggests that lines are characterized by the same slope and by no common intercept on the *Y*-axis, consistently with noncompetitive inhibtion. The following equation [14]:

$$J = \frac{J_{max} \cdot [\mathrm{Pro}]}{K_{app} \cdot (1 + ([\mathrm{Ala}]/K_i)) + [\mathrm{Pro}] \cdot (1 + ([\mathrm{Ala}]/K_i))}$$

was fitted to data and the following kinetic parameter were calculated: J_{max} (nmol/mg protein per min) = 9.65 ± 1.63 and 20.38 ± 1.43; K_{app} (mM) = 0.41 ± 0.14 and 0.23 ± 0.04; K_i (mM) = 5.42 ± 1.49 and 19.7 ± 4.81.

In brief, Na$^+$-dependent proline uptake was competitively inhibited by MeAIB and noncompetitively inhibited by alanine.

TABLE II

Inhibition of Na$^+$-dependent alanine proline and MeAIB uptakes in BBM vesicles from the intestine of the eel

This table summarizes the data from Figs. 2 and 3 (inhibition of proline uptake) Table I and Fig. 1 (inhibition of alanine uptake) and Fig. 4 (inhibition of MeAIB uptake).

Substrate	Inhibitor	Inhibition?
Proline	MeAIB	competitive
Proline	Alanine	noncompetitive
Alanine	MeAIB	no
Alanine	proline	yes
MeAIB	proline	yes
MeAIB	alanine	yes

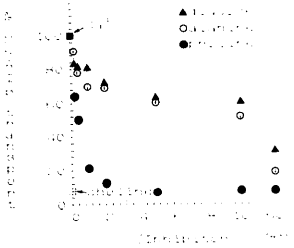

Fig. 4. Inhibition by L-proline, L-alanine and glucose of MeAIB uptake (5 s) in BBM vesicles from the eel intestine. Vesicles, prepared in a buffer containing (in mM) 300 mannitol, 50 KCl, 20 Hepes adjusted to pH 7.4 with Tris, were incubated in a medium containing (final concentration in mM) 100 mannitol, 50 KCl, 0.0224 valinomycin, 21.4 ethanol, 0.2 MeAIB, 20 Hepes adjusted to pH 7.4 with 100 Tris-NaCl, and glucose (triangles) or L-alanine (open circles) or L-proline (closed circles) ranging from 0 to 50. The control uptake values (0 mM inhibitor) were 3.95 ± 0.17 and 0.32 ± 0.04, respectively, in the presence (closed square) or absence (open square) of Na$^+$ ions.

Inhibition of MeAIB uptake

Fig. 4 reports data obtained when, in the presence of Na$^+$ ions in the extravesicular medium, the inhibition by proline, alanine and glucose of MeAIB (0.2 mM) uptake was measured. Glucose inhibited the MeAIB uptake (Fig. 4) as well as the alanine uptake (Fig. 1); this unspecific effect was very likely caused by collapse of the Na$^+$ gradient (see above).

A similar pattern of inhibition was shown by alanine. 2 mM proline completely inhibited MeAIB (0.2 mM) uptake, and about 50% of the total inhibition was observed with proline 0.4 mM.

Although Fig. 4, obtained with 1 substrate concentration, provides no information on the number of transport system(s) translocating MeAIB, we think we may cautiously conclude that the pattern of inhibition by alanine of proline (Table I) and MeAIB (Fig. 4) uptakes was similar.

Conclusions

The study of amino-acid carrier-mediated systems can be complicated by certain problems. By graphic and/or nonlinear regression procedures it is not possible to determine whether a substrate is translocated by one carrier or by two which are characterized by kinetic parameters within the same order of magnitude. A second example of somehow intricated experimental evidence is represented by Fig. 1. The fact that a substrate inhibits the uptake of a second substrate, is usually considered as demonstration that both substances are transported by the same carrier, although cases of noncompetitive inhibition have sometimes been reported [17,18]. Finally, any substrate cotransported with Na^+ may inhibit another Na^+/substrate cotransport by collapsing the Na^+ gradient; examples are the inhibitions by glucose of alanine (Fig. 1) and MeAIB (Fig. 4) uptakes. The Na^+-gradient decay can be experimentally reduced by using a low concentration of the potential inhibitor, but it will never be eliminated until an arsenal of amino-acid transport inhibitors has been developed.

According to Christensen's classification of Na^+-dependent amino-acid transport systems, MeAIB is the model substrate for the A system [5], while, according to Wright's group [6], MeAIB is the model substrate for a system specific for prolines and therefore called IMINO.

With this background, we used MeAIB as an inhibitor for both Na-dependent proline and alanine uptakes: if the three analogues were transported by the same system, the same K_i value for MeAIB inhibition of alanine and proline uptakes should result (Scholefield's test, [19]). Our experiments excluded the presence of a single carrier for the three substrates because: (1) MeAIB inhibited Na^+-dependent proline uptake (Fig. 2) but did not inhibit at all alanine uptake (Table I); (2) alanine inhibited Na^+-dependent proline uptake in a noncompetitive way (Fig. 2); (3) 1 mM alanine (close to the K_{app} value) would only slightly inhibit both Na^+-dependent proline and MeAIB uptakes (Table I, Fig. 4).

Our suggestion is that, on the BBM of the eel intestine, there are at least two distinct Na^+-dependent carriers for neutral amino acids: the first in charge of the great part of alanine transport, the second the great part of proline and MeAIB transport; moreover, the last carrier should possess a regulatory site accepting alanine. This hypothesis is consistent with the observed mutual inhibition between alanine and proline transports because Fig. 2 has shown that alanine inhibition of proline uptake was noncompetitive; unfortunately, further direct evidence, arising from proline noncompetitive inhibition of Na^+-dependent alanine uptake, could not be obtained.

According to our hypothesis, in BBM of the eel intestine, the behaviors of Na^+-dependent alanine and proline transports do not fit the following two models: (1) Christensen's classification, because the fraction of alanine uptake via the A system should be (and was not) inhibited by MeAIB and (2) Wright's model, because proline did not show a flux via the Na^+-dependent alanine carrier (NBB system) [20]. From the evolutionary point of view, it is a very attractive hypothesis that from the first pool of amino-acid plasma-membrane carriers (summarized by the Christensen's classification [5]), a second pool specific for BBM transport [6] evolved; the position of intestinal carriers of lower vertebrates, such as fish, could be intermediate. To verify this hypothesis, it is important, and experiments are in progress, to study the interaction of other amino acids and, in particular, phenylalanine, with the two Na^+-dependent transport agencies this paper has dealt with.

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