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INTESTINAL AMINO ACID ABSORPTION IN LEPIDOPTERAN LARVAE

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The characteristics of K^+-L-phenylalanine cotransport across brush border membrane vesicles isolated from the midgut of two lepidopteran larvae were studied. The amino acid is cotransported mainly with K^+ and Na^+, whereas other alkali metal cations are much less effective. The amino acid uptake displayed saturation kinetics with respect to external K^+ concentration and with respect to external amino acid concentration. In the latter case a free diffusion component was evident. The activation by K^+ involved an increase in J_{max} and a decrease in K_m. The involvement of alkali cations in amino acid absorption was also confirmed in the isolated midgut, by replacing K^+ with other monovalent cations in the luminal side only or on both sides. A possible model for amino acid absorption in the midgut of lepidopteran larvae, with K^+ as cotransported cation, is proposed and discussed.

Introduction

The midgut of lepidopteran larvae exhibits in vitro a transepithelial electrical potential difference across the wall of 100 mV or more, with the positive pole on the lumen side of the gut, due to an Na^+-independent active transport of K^+ from haemolymph to lumen [1–5]. Despite the extremely low Na^+ concentration (less than 5 mM) in the lumen content, midgut cells and haemolymph, the midgut actively transfers amino acids in a direction opposite to K^+ extrusion [6,7]. The transport takes place in the absence of luminal Na^+ [8], is not directly linked to the K^+ pumped towards the lumen, but does depend on the transepithelial electrical potential difference [9]. Finally, the amino acid transport mechanism appears to be located on the luminal membrane of the absorptive cell [10]. Moreover, using membrane vesicles obtained from *Philosamia cynthia* midgut, we have demonstrated that L-phenylalanine is cotransported with K^+ and, to a lesser extent, with Na^+ [11].

In the present paper a further characterization of amino acid uptake was performed using an improved membrane preparation. On the basis of experimental evidence obtained with vesicles as well as with the whole midgut isolated in vitro, a model for amino acid absorption in the midgut of lepidopteran larvae is proposed.

Material and Methods

Larvae in the fifth instar either of *P. cynthia* or of *Bombyx mori* were used. The larvae were reared in the laboratory and fed on *Ailanthus glandulosa* leaves and *Morus alba* leaves, respectively. The midgut was dissected from the larvae as a cylinder and deprived of the peritrophic membrane with enclosed intestinal content.

Brush border membrane vesicle preparation and transport experiments. Vesicles from *P. cynthia*

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

midgut were prepared by means of Ca^{2+} precipitation following the procedure of Schmitz et al. [12] as modified by Kessler et al. [13]. The midguts (up to 2 g fresh tissue) were carefully separated from malpighian tubules, opened lengthwise and repeatedly washed in an ice-cold saline solution of the following composition: 37 mM NaCl, 37 mM KCl, 150 mM mannitol, 5 mM EDTA (pH 7.5). The midguts were then crushed with a glass slide, and a 6% homogenate was made in 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5) at 4°C, using a glass teflon Thomas homogenizer, 9 strokes at 3000 rev./min. The homogenate was filtered through a double layer of cheese cloth, solid $CaCl_2$ was added to a final concentration of 10 mM, and the suspension thoroughly mixed and left for 15 min in the cold. The calcium-treated homogenate was then centrifuged at $3000 \times g$ for 15 min, the pellet obtained discarded, and the supernatant centrifuged at $27000 \times g$ for 30 min. The supernatant was then discarded and the pellet resuspended by means of the homogenizer (9 strokes at 3000 rev./min) in the buffer and spun down at $27000 \times g$ for 30 min. The final pellet, containing brush border membrane vesicles, was resuspended in the buffer by sucking the suspension 7 times through a stainless steel needle (25 G × 5/8) into a plastic syringe. Vesicles from *B. mori* midgut were prepared by repeating Ca^{2+} precipitation as described by Lucke et al. [14]. Transport experiments were performed by a rapid filtration technique, as already described [11]. Vesicles were incubated at room temperature in a mixture containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), the labelled amino acid and salt gradients as indicated in the legends of the tables and figures.

Determination of enzyme specific activities. Small aliquots of the crude homogenate and of vesicle suspension were withdrawn to measure enzyme activity and protein concentration. Disaccharidase activity was assayed according to Semenza and Von Balthazar [15] with sucrose and maltose as substrates and glucose oxidase/peroxidase method (Boehringer Test-combination). Alkaline phosphatase, aminopeptidase and γ-glutamyltransferase were determined with Boehringer kits, using, respectively, *p*-nitrophenylphosphate, L-leucine-*p*-nitroanilide and L-γ-glutamyl-3-carboxy-4-*p*-nitroanilide as substrates. Lactate dehydrogenase was assayed according to Bergmeyer and Bernt [16]. Protein determination was carried out according to Bradford [17], using a BioRad kit.

Flux measurements and cation concentration assays in the whole midgut. The midgut, excised from the larva, was mounted as a cylinder on an apparatus similar to that described by Nedergaard and Harvey [18]. The physiological solution used for perfusion contained 40 mM KCl, 2 mM $KHCO_3$, 37 mM $MgSO_4$, 9 mM $CaCl_2$, 141 mM sucrose and 10 mM L-phenylalanine (pH 7.4). In some experiments KCl was substituted by RbCl (40 mM), NaCl (40 mM) or choline chloride (40 mM). Both luminal and haemolymphatic solutions were stirred and aerated by forcing air through the inlets. 6 μCi/ml of (U-^{3}H)-labelled amino acid (Radiochemical Center, Amersham, U.K.) were added to the mucosal solution to measure the influx (lumen to haemolymph) and 1 μCi/ml of labelled amino acid was added to the haemolymph solution to measure the efflux (haemolymph to lumen). Samples were withdrawn from mucosal or haemolymph solution after an equilibration period of 30 min, which is necessary to obtain steady fluxes, and after 60 min. Radioactivity was tested by means of a liquid scintillation spectrometer (Tri-Carb Packard, Model 3385). At the end of the experiments, the exposed tissue was removed, put in a tared tube and weighed. Intracellular concentrations of K^+ and Na^+ were determined by use of a flame photometer and tissue values were corrected for ion concentration in the extracellular space as reported by Giordana and Sacchi [19]. The tissues were then stored in an oven (110°C) overnight to obtain the dry weight.

Results

The membrane preparation obtained from *P. cynthia* midgut and used for transport experiments was enriched in brush border marker enzymes. Actually, as can be seen in Table I, a 10-fold increase in enzyme activity was obtained for three out of the five tested typical brush border markers, i.e., alkaline phosphatase, leucine aminopeptidase and γ-glutamyltransferase. The small enrichment for sucrase is probably due to a different distribution of this enzyme on the plasma membranes of insect intestinal cells with respect to its localiza-

TABLE I

ENRICHMENT OF BRUSH BORDER MARKER ENZYMES IN MEMBRANE VESICLES FROM *P. CYNTHIA* MIDGUT

Enzyme activities are expressed as $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. Mean + S.E. of five determinations.

	Sucrase	Maltase	Alkaline phosphatase	Leucine aminopeptidase	γ-Glutamyl-transferase
Homogenate	0.215 ± 0.037	0.052 ± 0.012	0.475 ± 0.051	0.232 ± 0.011	0.0025 ± 0.0006
Vesicles	0.504 ± 0.114	0.045 ± 0.009	4.290 ± 0.069	2.232 ± 0.216	0.0270 ± 0.0030
Enrichment factor	2.3 ± 0.3	1.1 ± 0.6	9.0 ± 0.9	9.6 ± 0.8	14.3 ± 3.4

tion in mammalian enterocytes. This is supported by a lack of enrichment for maltase. No cytosolic contamination was present in the membrane fraction, since lactate dehydrogenase activity was undetectable in the vesicle suspension.

As far as L-phenylalanine uptake is concerned, these vesicles are endowed with the same properties that have been described in a previous work [11], namely the amino acid uptake is concentrative in the presence of a K^+ or Na^+ salt gradient and it is influenced by the transmembrane electrical potential difference, provided that K^+ or Na^+ is present. L-Phenylalanine is not the only amino acid accumulated inside the vesicles, since L-alanine initial uptake was also always higher than that found when equilibrium was attained (Table II). Similar results were obtained with membrane vesicles prepared from *B. mori* midgut (Table II). Despite a more complex method of preparation (see Materials and Methods), vesicles from *B. mori* always exhibited amino acid uptake per mg protein much smaller than that of *P. cynthia* (about 3.5-fold less both for L-alanine and L-phenylalanine, data not shown). This is due to the higher contamination with membranes other than luminal ones, as shown by electron microscope examination (unpublished data).

The amino acid uptake was strongly dependent on the nature of the cation that provided the driving force for the uptake. It is apparent from Table III that the amino acid (L-phenylalanine or L-alanine) cotransport was maximal with K^+ or Na^+, whereas it was lower with Li^+ or Rb^+ and absent with Cs^+ or choline.

The dependence of L-phenylalanine uptake on K^+ concentration was investigated by keeping the

TABLE II

MAXIMAL ACCUMULATION RATIO FOR L-PHENYLALANINE OR L-ALANINE IN THE PRESENCE OF A KSCN GRADIENT (100 mM OUTSIDE, ZERO INSIDE) INTO MEMBRANE VESICLES OF TWO DIFFERENT LEPIDOPTERAN LARVAE

	$[L\text{-phenylalanine}]_o$ [a] / $[L\text{-phenylalanine}]_e$ [b]	$[L\text{-alanine}]_o$ [a] / $[L\text{-alanine}]_e$ [b]
P. cynthia	3.17	2.81
B. mori	2.89	5.67

[a] Uptake (pmol/mg protein) at 3 min (overshoot value);
[b] Uptake (pmol/mg protein) at 60 min (equilibrium value).

TABLE III

EFFECT OF CATIONS ON L-PHENYLALANINE OR L-ALANINE UPTAKE INTO BRUSH BORDER MEMBRANE VESICLES OF TWO LEPIDOPTERAN LARVAE

The incubation medium was in all cases 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 1 mM L-phenylalanine (L-Phe) or L-alanine (L-Ala), 100 mM salt gradient. The amounts taken up during the first 3 min are indicated. Mean ± S.E. of three experiments.

Salt added	*P. cynthia* pmol L-Phe/mg protein	*B. mori* pmol L-Ala/mg protein
LiCl	3940 ± 833	203 ± 16
NaCl	5893 ± 940	1093 ± 120
KCl	7186 ± 600	1151 ± 340
RbCl	2890 ± 890	168 ± 23
CsCl	1660 ± 73	108 ± 9
Choline chloride	1515 ± 465	-

external Cl^- concentration constant by means of choline chloride, in order to minimize the variation in transmembrane electrical potential difference due to the decrease in the external KCl concentration. Fig. 1 shows the obtained results: the activation by K^+ of amino acid uptake displays saturation kinetics, but part of the L-phenylalanine uptake seems to be K^+ - independent, since the intercept on the vertical axis is not zero. Taking into account this K^+-independent component, the K^+ concentration that provides half maximum activation was calculated to be 24 mM.

Fig. 2 shows the kinetics of the uptake with respect to external L-phenylalanine concentration at saturating and nonsaturating K^+ concentrations. The experiments were performed keeping the external Cl^- concentration constant with addition of choline chloride. In the insert, a linear component is clearly evident at higher L-phenylalanine concentrations. Each experimental value was, therefore, corrected for this nonsaturable component, calculated on the basis of the linear part of the curve. The corrected values are shown in the Lineweaver-Burk plot indicated in Fig. 2; it is evident that both J_{max} and apparent K_m for L-phenylalanine are changed by K^+ concentration: a change from 100 mM to 10 mM K^+ caused a decrease in J_{max} value from 7416 ± 560 to 3960 ± 287 pmol · (15 s) $^{-1}$ · (mg protein) $^{-1}$, and an increase in apparent K_m from 0.51 ± 0.09 to 1.31 ± 0.12 mM.

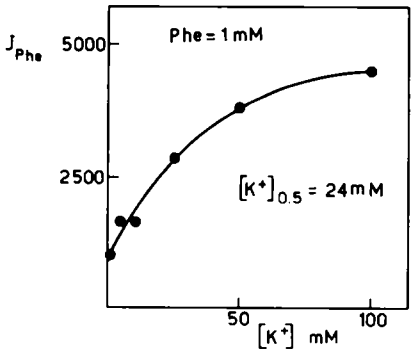

Fig. 1. Effect of KCl concentration on L-phenylalanine uptake in brush border membrane vesicles from *P. cynthia* midgut. The incubation medium contained 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 1 mM L-phenylalanine and KCl at the indicated concentrations outside, zero inside. Cl concentration was kept constant with addition of choline chloride. The amount taken up by the vesicles after 15 s is given.

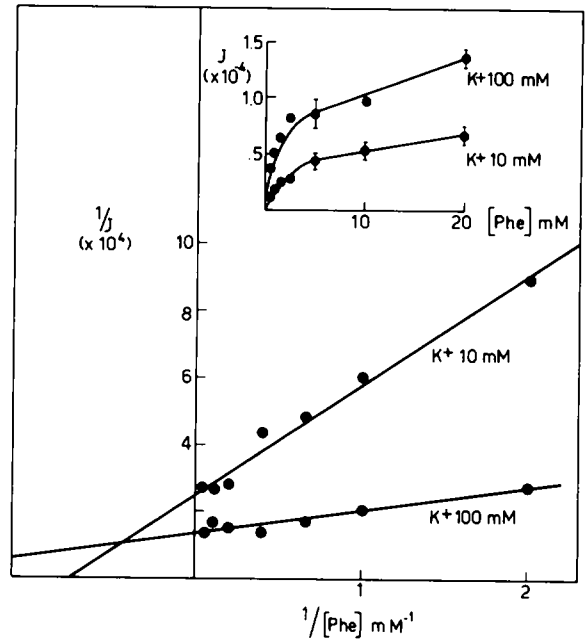

Fig. 2. Effect of external L-phenylalanine concentration on L-phenylalanine uptake in brush border membrane vesicles from *P. cynthia* midgut. The incubation medium contained 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 100 mM KCl outside (zero inside) or 10 mM KCl + 90 mM choline chloride outside (zero inside), and L-phenylalanine concentration outside as indicated. The amount taken up by the vesicles after 15 s is given. The bars indicate the S.E. of the mean.

The influence of alkali metal cations on L-phenylalanine absorption was also investigated in the whole intestine of *B. mori* and *P. cynthia* isolated in vitro (Table IV). The amino acid influx $J_{l \cdot h}$ (lumen to haemolymph) and efflux $J_{h \cdot l}$ (haemolymph to lumen) were measured either in the presence of K^+ on both sides of the tissue (control) or by substituting K^+ with other monovalent cations on the luminal side only or on both sides. In order to keep the transepithelial electrical potential difference unaltered, the cations were chosen taking into account the known specificity of the midgut K^+ pump, which scarcely discriminates between K^+ and Rb^+ [20,21]. In a first set of experiments, KCl was substituted by equal amounts of RbCl on both sides of the tissue, in order to avoid any enrichment in luminal K^+.

TABLE IV

UNIDIRECTIONAL FLUXES OF L-PHENYLALANINE IN ISOLATED MIDGUTS OF *B. MORI* AND *P. CYNTHIA* MEASURED IN DIFFERENT IONIC ENVIRONMENTS

V_{ms}, transepithelial electrical potential difference (mV) after 30 min; lumen positive with respect to haemolymph. Fluxes are expressed as $\mu mol \cdot h^{-1} \cdot (g\ dry\ weight)^{-1}$; J_{l-h} = lumen to haemolymph; J_{h-l} = haemolymph to lumen; J_{net} = mean J_{l-h} − mean J_{h-l}. Mean ± S.E.; number of experiments in parenthesis; P was calculated with the t test. The experiments were carried out in October (A, upper part) and in late November (B, lower part).

		V_{ms}	J_{l-h}	J_{h-l}	J_{net}	P
A. Control [a]	*B. mori*	78.1 ± 5.2 (9)	116.2 ± 13.8 (6)	27.1 ± 2.6 (4)	89.1 ± 17.3 (10)	-
	P. cynthia	57.4 ± 5.6 (8)	61.4 ± 11.0 (5)	10.4 ± 0.6 (4)	50.0 ± 12.4 (9)	-
A. RbCl [b]	*B. mori*	69.9 ± 5.9 (9)	53.2 ± 6.2 (6)	31.7 ± 8.6 (4)	21.5 ± 10.3 (10)	<0.01
	P. cynthia	64.7 ± 4.9 (9)	29.0 ± 6.2 (5)	10.4 ± 0.6 (4)	18.6 ± 7.0 (9)	<0.05
B. Control [a]	*B. mori*	89.3 ± 6.3 (6)	82.6 ± 9.4 (2)	27.3 ± 2.8 (4)	55.5 ± 7.1 (6)	-
B. NaCl [c]	*B. mori*	69.6 ± 8.7 (5)	66.4 ± 15.3 (3)	29.1 ± 3.1 (5)	37.3 ± 11.8 (8)	n.s.
B. ChCl [d]	*B. mori*	99.2 ± 4.8 (7)	41.6 ± 5.5 (4)	28.3 ± 6.0 (3)	13.3 ± 7.8 (7)	<0.01

[a] Midguts perfused with a solution containing 40 mM KCl on both sides.

[b] Midguts perfused with a solution containing 40 mM RbCl instead of KCl on both sides.

[c] Midguts perfused with a solution containing 40 mM NaCl instead of KCl on the lumen side and 40 mM KCl on the haemolymph side.

[d] Midguts perfused with a solution containing 40 mM choline chloride (ChCl) instead of KCl on the lumen side and 40 mM KCl on the haemolymph side.

Under these conditions, the amino acid influx was reduced and the net flux was strongly inhibited. In a second set of experiments, performed on *B. mori* isolated midguts only, luminal KCl was replaced by equal amounts of NaCl or choline chloride, leaving KCl concentration on the haemolymph side unaltered. Under these conditions luminal K^+ concentration increased to 5 mM, although the luminal volume was raised to 3 ml, since the net rate of K^+ extrusion is very high, up to 50 $\mu equiv. \cdot cm^{-2} \cdot h^{-1}$ [22]. In the presence of luminal Na^+ the amino acid net flux was moderately reduced to a value that did not significantly differ from the control, whereas it was significantly lowered in the presence of the impermeable cation choline.

In all tested conditions only the influx was reduced, whereas no change in the effluxes was recorded. The different values of J_{l-h} and J_{net} in control experiments shown in Table IV are due to the different period of the autumn in which larvae were reared.

Discussion

The membrane preparation used in the present work was enriched with marker enzymes of brush border, the localization of which has been demonstrated mainly for renal and intestinal cells of vertebrates [23]. Although little is known about the enzymatic pattern of insect brush border, the same localization has been found for the studied enzymes (see γ-glutamyltransferase [24]). The marker enzyme for renal and enterocyte basolateral membranes, the $(Na^+ + K^+)$-ATPase, was not considered since this enzyme is not present in the midgut of lepidopteran larvae [25].

With this vesicle preparation, the dependence of L-phenylalanine uptake by alkali cations has been investigated. The specificity pattern of amino acid cotransport found in lepidopteran midgut compared to that in mammalian intestine appears to be shifted to alkali cations of higher molecular weight. Actually, in mammals the effective cation in cotransport mechanism is Na^+, followed by Li^+ [26], whereas in lepidoptera the most effective cation is K^+, followed by Na^+. The uptake of L-phenyalanine observed in the absence of any

alkali cation (Table III and Fig. 1) could be due to aspecific binding of the amino acid to membranes, since, at equilibrium, about 15% of total L-phenylalanine uptake is not due to a transport into an osmotically active space [11]. Besides, the possibility of a K^+-independent L-phenylalanine uptake cannot be ruled out, as it can be inferred from the diffusion component shown in Fig. 2.

The dependence of amino acid absorption from luminal monovalent cations was also studied in the whole midgut perfused in vitro (Table IV). Such a dependence is not as easy to study as in vesicles, since the removal of K^+ from the haemolymph side causes a rapid decrease in the transepithelial electrical potential difference, which influences per se the absorption of amino acids [9]. Therefore, K^+ was replaced by Rb^+ on both sides of the tissue and by Na^+ or choline on the luminal side only. The obtained data agree with the cotransport specificity found in membrane vesicles and they allow us to conclude that the transepithelial electrical potential difference is a necessary but not the sole condition for the transport, since the presence of K^+ or Na^+ is also required.

However, the only cation that most likely plays an effective role in vivo is K^+, owing to its luminal concentration in comparison to that of Na^+ [19]. As a matter of fact, the intestine of the lepidopteran larvae plays a specific homeostatic role to keep haemolymph K^+ concentration constant and low [1]. This is accomplished by an electrogenic K^+-pump, which generates a lumen-positive transepithelial electrical potential difference of 100–150 mV and which, on the basis of all available evidence [5], is located on the luminal membrane of a specialized kind of cell, the goblet cell [27]. Absorption of ions and nutrients takes place in columnar cells [28], whose morphology is similar to other absorptive cells. In columnar cells the transmembrane electrical potential difference measured across the basolateral membrane (V_s) is negative with respect to the haemolymph compartment, since recorded values of -34.1 ± 1.2 mV (mean ± S.E. of 69 impalements) in *B. mori* and -31.9 ± 0.8 mV (mean ± S.E. of 95 impalements) in *P. cynthia* have been obtained (Monticelli, G., personal communication). Similar values have been reported for other species of Lepidoptera [29–31]. Moreover, the intracellular K^+ activity can be estimated by its cellular concentration and assuming an activity coefficient of 0.8 [32]. These data are summarized in Table V and they allow calculation of the K^+ electrochemical potential difference ($\Delta\bar{\mu}_K$) across the luminal membrane of columnar cells. As is apparent, whenever the calculated $\Delta\bar{\mu}_K$ is largely negative, the amino acid J_{net} is high (in vivo and in vitro control conditions), whereas J_{net} is inhibited when $\Delta\bar{\mu}_K$ is reduced (in vitro in the

TABLE V

PRESUMED POTASSIUM ACTIVITY AND POTASSIUM ELECTROCHEMICAL POTENTIAL DIFFERENCES ACROSS BRUSH BORDER MEMBRANE OF THE COLUMNAR MIDGUT CELLS OF *B. MORI* AND *P. CYNTHIA*

Potassium activity (a_K) is expressed as mmol/liter cell water. Intracellular a_K was calculated assuming an activity coefficient of 0.8 [32]. Extracellular a_K was calculated assuming an activity coefficient of 0.77 [35]. V_m represents the difference between the transepithelial electrical potential difference (V_{ms}) reported in Table IV and the transerosal electrical potential difference (V_s) reported in the text, since $V_m = V_{ms} - V_s$.

	Condition	a_K		V_m (mV)	$\Delta\bar{\mu}_K$ (cal·mol^{-1})
		Intracellular	Extracellular		
B. mori	in vitro; KCl [a]	145	30.8	108	−1594
P. cynthia	in vitro; KCl [a]	152	30.8	87.4	−1091
B. mori	in vitro; RbCl [b]	64	1.5	99.9	−128
B. mori	in vitro; NaCl [c]	128.2	3.9	100.0	−282
B. mori	in vivo	158	115	150	−3278
P. cynthia	in vivo	194	152	150	−3320

[a,b,c] As in Table IV.

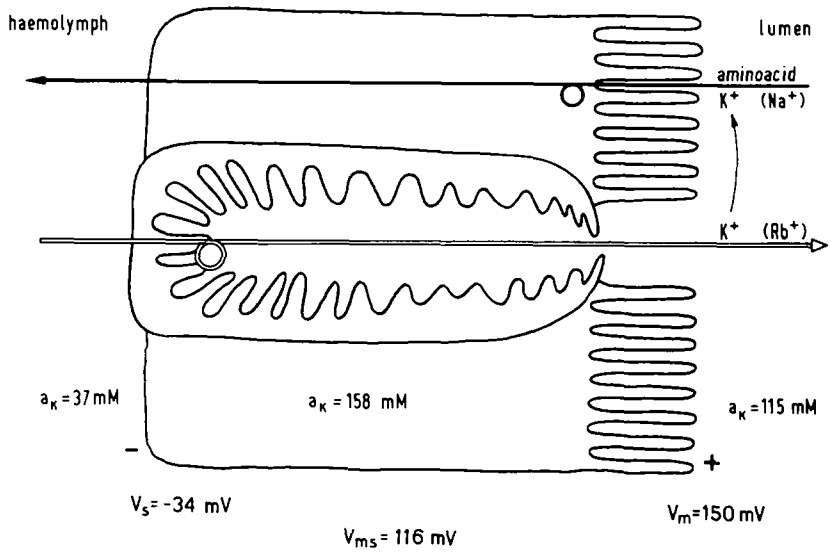

Fig. 3. Model for amino acid absorption across intestinal cells of lepidopteran larvae. A goblet cell between two columnar cells are indicated. The black arrow indicates the amino acid carrier, the white arrow indicates the K^+- pump. V_s, transerosal electrical potential difference; V_m, transmucosal electrical potential differences; V_{ms}, transepithelial electrical potential difference. Since the cell interior is negative with respect to external compartments, polarities can be defined as follows: V_s = cell → haemolymph; V_m = lumen → cell; V_{ms} = lumen → haemolymph. Values of electrical potential differences and potassium activities refer to *B. mori* in vivo.

presence of Rb^+ or choline). However, by replacing luminal K^+ with Na^+, the J_{net} of the amino acid is only slightly diminished, despite the low value of $\Delta\bar{\mu}_K$. However, in this case the electrochemical potential difference for Na^+ is largely negative ($\Delta\bar{\mu}_{Na} = -2000$ cal · mol^{-1}, data not shown) and this energy can be coupled to amino acid transport, since Na^+ is effective as a cotrasported cation.

It is also apparent from Table V that the negative value of $\Delta\bar{\mu}_K$ is completely dependent on the electrical component, since the chemical component is positive. Therefore, it can be predicted that under short-circuit conditions the amino acid net transport should be drastically reduced. As a matter of fact, in *Hyalophora cecropia* a drop in the net transport of α-aminoisobutyric acid of 70% with respect to the control (in open circuit condition) has been observed [9].

On the basis of the evidence reported in this paper, a possible model for amino acid absorption in the midgut of lepidopteran larvae can be proposed (Fig. 3): under physiological conditions, the driving force for amino acid absorption is supplied by the K^+ electrochemical gradient maintained by the luminally directed K^+- pump. In parenthesis (Fig. 3) are indicated those ions which in vitro can efficiently replace K^+ in the ionic pump and in the cotransport mechanism: i.e. Rb^+ and Na^+, respectively. But it is evident that the whole machinery does work in vivo only in the presence of K^+, that is, the ion shared by both components. The model, mutatis mutandis, corresponds to that proposed by Crane [33] and Schultz and Zalusky [34] for glucose and amino acid absorption in mammalian intestine. However, in lepidopteran intestinal mucosa, possibly as a result of a broadening of the specificity of the carrier, K^+ and not Na^+ is the cation cotransported with the amino acid, and two different kinds of cells, not two different portions of the plasma membrane of the same cell, cooperate in ionic homeostasis and metabolite absorption.

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