LEUCINE UPTAKE IN BRUSH-BORDER MEMBRANE VESICLES FROM THE MIDGUT OF A LEPIDOPTERAN LARVA, PHILOSAMIA CYNTHIA

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Summary

A potassium- or sodium-activated cotransport of leucine occurs in brush-border membrane vesicles prepared from the midgut of larvae of Philosamia cynthia (Drury). The potassium chemical gradient can drive a twofold accumulation of leucine, which is greatly increased under experimental conditions that presumably provide an electrical potential difference ($\Delta \psi$).

Kinetic parameters show that leucine transport is improved by these conditions and by a pH gradient similar to that occurring in vivo. However, these gradients cannot drive an intravesicular accumulation of leucine in the absence of potassium. The potassium-dependence of leucine uptake shows that 20% of the transport is potassium-independent and that $K_{50}$ and $V_{\text{max}}$ are 30.3±3.2 mmol l$^{-1}$ and 2584±148 pmol s$^{-1}$mg$^{-1}$ protein, respectively. The potassium-independent component of leucine transport is also carrier-mediated and some evidence is reported suggesting that the same carrier can cross the membrane as binary (carrier and leucine) or ternary (carrier, leucine and potassium) complexes, each having a different mobility.

Introduction

Several transport systems with different amino acid and cation specificities are located on the brush border of the midgut columnar cells of Philosamia cynthia larvae (Hanozet et al. 1989; Giordana et al. 1989). These voltage-dependent cotransport mechanisms couple in vivo amino acid and potassium influxes (Giordana et al. 1982). The driving force for this process is mostly provided by the high electrical potential difference present at the luminal border (cell interior negative to the lumen) generated by a potassium pump (Harvey et al. 1983; Dow et al. 1984; Moffet and Koch, 1988a,b). Indeed, potassium is the main monovalent

Key words: leucine uptake, amino acid transport, brush-border membrane vesicles, insect midgut.
cation present *in vivo* in this peculiar experimental substrate, sodium ion concentration being very low in all larval tissues (Giordana and Sacchi, 1978) except the central nervous system (Abbott and Treherne, 1977; Monticelli *et al.* 1985). Nevertheless, in most cases, sodium can efficiently replace *in vitro* potassium in the cotransport mechanism (Giordana *et al.* 1989).

In this work, leucine transport in purified brush-border membrane vesicles from *Philosamia cynthia* larvae has been studied. Leucine is transported by a system which is responsible for the transfer of most neutral amino acids (Hanozet *et al.* 1989; Giordana *et al.* 1989) and this paper adds new information on the characteristics of this transport system.

**Materials and methods**

*Preparation of brush-border membrane vesicles*

Fifth-instar larvae of *Philosamia cynthia* were used. The midguts were dissected from the larvae, the peritrophic membrane with the enclosed intestinal content and the Malpighian tubules were removed. The tissues were rinsed in ice-cold solution (150 mmol l⁻¹ sucrose, 75 mmol l⁻¹ KCl, pH 7.4) and frozen in liquid nitrogen. The frozen tissues were stored at −80°C for periods not exceeding 6 months. Samples of the frozen midguts (about 2 g fresh mass) were rapidly thawed at 40°C and then used to prepare the brush-border membrane vesicles (BBMV). The preparation was as described in a previous paper (Giordana *et al.* 1982). When preloading of the vesicles was necessary, the pellet from the second centrifugation step and the final pellet were resuspended in a medium having the composition reported in the legends of the figures and the tables. The final membrane pellet was resuspended at a protein concentration of 10–15 mg ml⁻¹, determined according to Bradford (1976) with a Bio-Rad kit, using bovine serum albumin as standard.

*[^3H]Leucine uptake*

Transport experiments were performed in quadruplicate by a rapid filtration technique, as described by Hanozet *et al.* (1980). Incubation times shorter than 10 s were carried out with an automated apparatus consisting of a timer which controlled both a shaker (Vibrofix VFI, Janke & Kunkel Ika Werk, Staufen, FRG) and an injector (Automatic Dispenser, Oxford, Athy, Ireland). A drop of the cocktail (10 μl, 40 μl or 190 μl) and a drop of the BBMV suspension (10 μl) were placed at the bottom of a tube fitted into the shaker. The incubation was started by shaking the test tube for 0.5 s at about 2000 revs min⁻¹, so that the two drops rapidly mixed. To stop the incubation, 2 ml of ice-cold stop solution was automatically injected into the test tube by the dispenser at the maximum speed. The sample was then filtered and radioactivity associated with the filters was measured with a Packard scintillation counter, model 300 C.

The uptake of leucine was linear up to 12 s and incubations of 7 s were considered reliable for measuring initial uptake.
When used, valinomycin or carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP) was added from stock solutions in ethanol, so that the ethanol concentration in the incubation mixture did not exceed 0.5%.

**Materials**

L-[U-3H]Leucine was obtained from the Radiochemical Centre (Amersham International, Amersham, UK). Valinomycin, N-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid (Hepes) and 2-(N-morpholino)ethanesulphonic acid (Mes) were obtained from Boehringer (Mannheim, FRG). FCCP was obtained from Sigma (St Louis, MO, USA). All other reagents were analytical grade products from BDH (Chemicals Ltd, Poole, England).

**Results**

The uptake of leucine by BBMV prepared from frozen midguts showed, in the presence of a potassium gradient, a high peak that was about eightfold higher than the equilibrium value (Fig. 1). Sodium could also energize the cotransport mechanism, while in the absence of any salt gradient, leucine uptake was merely equilibrative. The system involved in leucine transport therefore does not seem to discriminate between sodium and potassium. Lithium is also effective to a certain extent (Table 1).

Table 2 shows the effect of anion substitutions on leucine uptake. Assuming that the relative permeabilities of these anions across the vesicle membrane are similar to those measured in vertebrate membranes (as seems likely from preliminary data).

![Fig. 1. Time courses of leucine uptake in the presence of a sodium (●) or potassium (▲) gradient. BBMV conditions at zero time. In: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes–Tris, pH 7.4; out: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes–Tris, pH 7.4, 0.5 mmol l⁻¹ L-[3H]leucine (30 µCi ml⁻¹), 100 mmol l⁻¹ KSCN (▲), 100 mmol l⁻¹ NaSCN (●), no salt (■). Each point represents the mean ± s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbol used.](image-url)
Table 1. Effect of monovalent cations on leucine uptake

<table>
<thead>
<tr>
<th>Salt</th>
<th>10 s</th>
<th>1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMACl</td>
<td>375±23</td>
<td>–</td>
</tr>
<tr>
<td>LiCl</td>
<td>401±19</td>
<td>1316±52</td>
</tr>
<tr>
<td>NaCl</td>
<td>1026±77</td>
<td>2309±144</td>
</tr>
<tr>
<td>KCl</td>
<td>779±30</td>
<td>2275±58</td>
</tr>
<tr>
<td>RbCl</td>
<td>336±59</td>
<td>407±70</td>
</tr>
</tbody>
</table>

BBMV conditions at zero time. In: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes-Tris, pH 7.4; out: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes-Tris, pH 7.4, 0.5 mmol l⁻¹ L-[³H]leucine (30 μCi ml⁻¹), 100 mmol l⁻¹ of the indicated salt.

Uptakes are expressed as pmol mg⁻¹ protein.
Mean±s.e. of an experiment performed in triplicate.
TMA, tetramethylammonium.

Table 2. Effect of anions on leucine uptake

<table>
<thead>
<tr>
<th>Salt</th>
<th>10 s</th>
<th>1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSCN</td>
<td>1241±34</td>
<td>3236±43</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1052±17</td>
<td>3136±29</td>
</tr>
<tr>
<td>KCl</td>
<td>779±30</td>
<td>2275±58</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>818±155</td>
<td>2476±39</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>847±129</td>
<td>2167±122</td>
</tr>
</tbody>
</table>

BBMV conditions at zero time. In: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes-Tris, pH 7.4; out: 100 mmol l⁻¹ mannitol, 10 mmol l⁻¹ Hepes-Tris, pH 7.4, 0.5 mmol l⁻¹ L-[³H]leucine (30 μCi ml⁻¹), 100 mmol l⁻¹ of the indicated salts except K₂SO₄, which was 50 mmol l⁻¹.

Uptakes are expressed as pmol mg⁻¹ protein.
Mean±s.e. of an experiment performed in triplicate.

Fluorimetric measurements performed with a voltage-sensitive cyanine dye), leucine uptake seems to be sensitive to the transmembrane electrical potential difference (Δψ). As a matter of fact, higher uptake values were found in the presence of highly permeable anions such as SCN⁻ and NO₃⁻, compared with those found in the presence of poorly permeable anions such as SO₄²⁻ and gluconate. Cl⁻ also seems to have a low permeability through these membranes, in agreement with data previously reported (Hanozet et al. 1984).

To investigate the mutual contribution to the cotransport mechanism of the chemical or the electrical component of the K⁺ electrochemical gradient, leucine uptake was measured: (i) in the presence of a presumed Δψ generated by a gradient of a salt of an impermeant cation such as choline with a very permeant anion such as NO₃⁻ (50 mmol l⁻¹ outside, 0 mmol l⁻¹ inside), in the absence of the driver cation; (ii) in the presence of a K⁺ electrochemical gradient (50 mmol l⁻¹ KNO₃ outside, 0 mmol l⁻¹ inside); (iii) in the presence of a K⁺ chemical gradient (50 mmol l⁻¹ KNO₃ and 50 mmol l⁻¹ choline nitrate outside, 100 mmol l⁻¹ choline nitrate inside).
Leucine uptake in BBMV from insect midgut

Fig. 2. Time course of leucine uptake in the presence of $\Delta \psi$ (■), or of an electrochemical (●) or chemical (▲) potassium gradient. BBMV conditions at zero time. (■) in: 300 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4; out: 200 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4, 0.5 mmol l$^{-1}$ L-[^3]H]leucine (30 μCi ml$^{-1}$), 50 mmol l$^{-1}$ choline nitrate. (●) in: 300 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4; out: 200 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4, 0.5 mmol l$^{-1}$ L-[^3]H]leucine (30 μCi ml$^{-1}$) and 50 mmol l$^{-1}$ KNO$_3$. (▲) in: 100 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4, 100 mmol l$^{-1}$ choline nitrate; out: 100 mmol l$^{-1}$ mannitol, 10 mmol l$^{-1}$ Hepes-Tris, pH 7.4, 0.5 mmol l$^{-1}$ L-[^3]H]leucine (30 μCi ml$^{-1}$), 50 mmol l$^{-1}$ KNO$_3$ and 50 mmol l$^{-1}$ choline nitrate. Each point represents the mean±s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbol used.

nitrate inside). In this last case the membrane potential, generated by potassium diffusion, was short-circuited by the highly permeant anion NO$_3^-$, which was present at the same concentration on both sides of the vesicles (Kessler and Semenza, 1983). As shown in Fig. 2, the uptake of leucine was equilibrative in the presence of a simple electrical potential difference and it was concentrative in the presence of a potassium electrochemical gradient, with an accumulation of fivefold the equilibrium value. Conversely, leucine accumulation driven by the potassium chemical gradient, in the absence of $\Delta \psi$, was considerably lower (about twofold).

Initial leucine uptake (at 7 s) as a function of cis (external) leucine concentration was measured in the presence of either a chemical or an electrochemical potassium gradient obtained as indicated previously for Fig. 2. Fig. 3 reports the Eadie–Hofstee plot of the data, after subtraction of a linear component observed in the presence of choline. The kinetic parameters reported in Table 3A show that the absence of $\Delta \psi$, was considerably lower (about twofold).

The kinetics of leucine transport was further investigated in experimental conditions more similar to those occurring in vivo across the apical membrane of columnar cells (Dow, 1984, 1986), i.e. in the presence of $\Delta \psi$ and of a pH gradient 7.4 in/8.8 out) (Fig. 4). In all conditions a cis potassium concentration of
Initial uptake/[leucine]

Fig. 3. Kinetics of leucine initial uptake as a function of cis leucine concentration in the presence of either a chemical or an electrochemical potassium gradient. Eadie-Hofstee plot of the data after subtraction of a linear component. BBMV conditions at zero time. (▲) in: 100 mmol l\(^{-1}\) mannitol, 10 mmol l\(^{-1}\) Hepes-Tris, pH 7.4, 160 mmol l\(^{-1}\) choline nitrate; out: 100 mmol l\(^{-1}\) mannitol, 10 mmol l\(^{-1}\) Hepes-Tris, pH 7.4, 80 mmol l\(^{-1}\) KNO\(_3\), 80 mmol l\(^{-1}\) choline nitrate, L-[\(^3\)H]leucine (80 µCi ml\(^{-1}\)). (●) in: 420 mmol l\(^{-1}\) mannitol, 10 mmol l\(^{-1}\) Hepes-Tris, pH 7.4; out: 260 mmol l\(^{-1}\) mannitol, 10 mmol l\(^{-1}\) Hepes-Tris, pH 7.4, 80 mmol l\(^{-1}\) KNO\(_3\), L-[\(^3\)H]leucine (80 µCi ml\(^{-1}\)). Kinetic parameters were obtained by linear regression analysis according to the least-squares method. Each point represents the mean±s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbols used.

Table 3. Effect of pH gradient, K\(^+\) gradient and presumed Δψ modulations on leucine kinetic parameters

<table>
<thead>
<tr>
<th>Δψ</th>
<th>ΔpH</th>
<th>ΔK(^+)</th>
<th>(K_m) (mmol l(^{-1}))</th>
<th>(V_{max}) (pmol 7 s(^{-1}) mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>+</td>
<td>0.68±0.06</td>
<td>1650±210</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td></td>
<td>0.34±0.03</td>
<td>1942±68</td>
</tr>
<tr>
<td>B</td>
<td>−</td>
<td>+</td>
<td>0.38±0.03</td>
<td>3023±116</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.26±0.02</td>
<td>6599±255</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.55±0.04</td>
<td>3648±101</td>
</tr>
</tbody>
</table>

Means±s.e.
A, experimental conditions as reported in Fig. 3.
B, experimental conditions as reported in Fig. 4.
80 mmol l\(^{-1}\) was present and sulphate was used as counterion to avoid any interference with the genesis of \(\Delta \psi\). \(\Delta \psi\) was turned on/off by addition/no addition of the protonophore FCCP to the incubation medium. The \(K^+\) gradient was abolished by preloading the vesicles with 40 mmol l\(^{-1}\) \(K_2\)SO\(_4\). Table 3B reports the kinetic parameters calculated from the Eadie-Hofstee plot of the data displayed in Fig. 4. The protonophore, i.e. \(\Delta \psi\), causes a large increase in \(V_{\text{max}}\) and a decrease in \(K_m\). Conversely, 80 mmol l\(^{-1}\) \textit{trans} potassium caused a 45% reduction in \(V_{\text{max}}\) and a twofold increase in \(K_m\) compared to the control (0 mmol l\(^{-1}\) \textit{trans} potassium). Comparing the kinetic parameters reported in the first and second lines of sections A and B of Table 3, it is apparent that pH affects the function of this carrier mechanism since \(V_{\text{max}}\) values are much higher in the presence of a \(\Delta \text{pH}\). Furthermore, a pH gradient causes a higher intravesicular accumulation of leucine compared with those observed at nearly neutral (7.4 in/7.4 out) or basic (8.9 in/8.9 out) pH values (Fig. 5).

The dependence of leucine transport on the \textit{cis} potassium concentration was

Fig. 4. Kinetics of leucine initial uptake as a function of \textit{cis} leucine concentration in the presence of a \(\Delta \psi\), a pH gradient or a potassium gradient. Eadie-Hofstee plot of the data reported in the inset. BBMV conditions at zero time. (■) in: 160 mmol l\(^{-1}\) mannitol, 90 mmol l\(^{-1}\) Hepes, 45 mmol l\(^{-1}\) Tris, pH 7.4, 40 mmol l\(^{-1}\) \(K_2\)SO\(_4\); out: 184 mmol l\(^{-1}\) mannitol, 57 mmol l\(^{-1}\) Tris, pH 8.8, 40 mmol l\(^{-1}\) \(K_2\)SO\(_4\), 0.05–5 mmol l\(^{-1}\) \textit{L-[\(^3\)H]leucine (80 µCi ml\(^{-1}\)), 0.1 mmol l\(^{-1}\) FCCP. (●) in: 160 mmol l\(^{-1}\) mannitol, 90 mmol l\(^{-1}\) Hepes, 45 mmol l\(^{-1}\) Tris, pH 7.4, out: 184 mmol l\(^{-1}\) mannitol, 57 mmol l\(^{-1}\) Tris, pH 8.8, 40 mmol l\(^{-1}\) \(K_2\)SO\(_4\), 0.05–5 mmol l\(^{-1}\) \textit{L-[\(^3\)H]leucine (80 µCi ml\(^{-1}\)), 0.1 mmol l\(^{-1}\) FCCP. (▲) in: 160 mmol l\(^{-1}\) mannitol, 90 mmol l\(^{-1}\) Hepes, 45 mmol l\(^{-1}\) Tris, pH 7.4, out: 184 mmol l\(^{-1}\) mannitol, 57 mmol l\(^{-1}\) Tris, pH 8.8, 40 mmol l\(^{-1}\) \(K_2\)SO\(_4\), 0.05–5 mmol l\(^{-1}\) \textit{L-[\(^3\)H]leucine (80 µCi ml\(^{-1}\)). Kinetic parameters were obtained by linear regression analysis according to the least-squares method. Each point represents the mean±s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbols used.
investigated under the physiological conditions reported above, i.e. in the presence of a constant $\Delta \psi$ generated by a pH gradient (7.4 in/8.8 out) and FCCP. Iso-osmolarity was maintained with mannitol. The activation of leucine uptake induced by potassium displayed saturation kinetics (Fig. 6, inset), but part of leucine uptake was potassium-independent, since the intercept on the vertical axis was not zero. After subtraction of the potassium-independent component, the corrected values were plotted according to Eadie–Hofstee (Fig. 6), yielding a $K_{50}$ of 30.3±3.2 mmol l$^{-1}$ and a $V_{\text{max}}$ of 2584±148 pmol s$^{-1}$ mg$^{-1}$ protein. The potassium concentration in the luminal fluid in vivo is about 200 mmol l$^{-1}$ (Giordana and Sacchi, 1978), so the transporter can be considered fully saturated for potassium in vivo.

Since an appreciable uptake of leucine was detectable at 0 mmol l$^{-1}$ cis potassium (Fig. 6, inset), kinetic experiments were performed in the absence of potassium. Fig. 7 shows the effect of $\Delta \text{pH}$ with and without FCCP on the

![Fig. 5. Time courses of leucine accumulation at $\text{pHi}=7.4$, $\text{pHo}=8.8$ (●); $\text{pHi}=\text{pHo}=7.4$ (▲); $\text{pHi}=\text{pHo}=8.8$ (■). BBMV conditions at zero time. (●) in: 160 mmol l$^{-1}$ mannitol, 90 mmol l$^{-1}$ Hepes, 45 mmol l$^{-1}$ Tris, pH 7.4; out: 184 mmol l$^{-1}$ mannitol, 18 mmol l$^{-1}$ Hepes, 57 mmol l$^{-1}$ Tris, pH 8.8, 50 mmol l$^{-1}$ K$_2$SO$_4$, 0.5 mmol l$^{-1}$ L-[3H]leucine (30 $\mu$Ci ml$^{-1}$). (▲) in: 160 mmol l$^{-1}$ mannitol, 90 mmol l$^{-1}$ Hepes, 45 mmol l$^{-1}$ Tris, pH 7.4; out: the same solution present inside plus 100 mmol l$^{-1}$ KSCN, 0.5 mmol l$^{-1}$ L-[3H]leucine (30 $\mu$Ci ml$^{-1}$). (■) in: 184 mmol l$^{-1}$ mannitol, 18 mmol l$^{-1}$ Hepes, 57 mmol l$^{-1}$ Tris, pH 8.8; out: the same solution present inside plus 100 mmol l$^{-1}$ KSCN, 0.5 mmol l$^{-1}$ L-[3H]leucine (30 $\mu$Ci ml$^{-1}$). Each point represents the mean±s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbols used.]
potassium-independent uptake of leucine as a function of cis leucine concentration. In all cases, leucine uptake displayed saturation kinetics, indicating that the potassium-independent component of leucine transport is also carrier-mediated. The uptake values, plotted according to Eadie–Hofstee, yield the $K_m$ and $V_{\text{max}}$ values reported in Table 4. It is apparent that the presence of a pH gradient causes an increase in $V_{\text{max}}$ and a decrease in $K_m$, as observed in the presence of potassium (Table 3A,B). The effect of $\Delta \psi$ on leucine kinetic parameters in the absence of potassium (third line of Table 4) is lower than that observed in the presence of potassium.

The effect of 2 mmol$^{-1}$ $\text{trans}$ (internal) leucine on leucine kinetic parameters was measured in the absence of potassium. To lower the external concentration of leucine to 0.1 mmol$^{-1}$, the vesicles were diluted 20-fold into the incubation medium. Table 5 shows that $\text{trans}$ leucine exerted a trans-stimulation, causing a 50% increase of $V_{\text{max}}$ and a modest lowering of $K_m$. This effect can be explained if the binary complex (carrier and amino acid) has a higher mobility than the empty carrier. A similar effect was also observed in the presence of both $\Delta \psi$ and a pH gradient. In this case, the effect of $\text{trans}$ leucine was essentially a reduction of $K_m$ (data not shown).

Does leucine transport occur via two different transporters (one potassium-dependent and the other potassium-independent) or through a single carrier that crosses the membrane as a binary or ternary complex? In an attempt to answer this

Fig. 6. Kinetics of leucine uptake as a function of cis potassium concentration. Eadie–Hofstee plot of the data reported in the inset. BBMV conditions at zero time. In: 160 mmol$^{-1}$ mannitol, 90 mmol$^{-1}$ Hepes, 45 mmol$^{-1}$ Tris, pH 7.4; out: 184 mmol$^{-1}$ mannitol, 57 mmol$^{-1}$ Tris, pH 8.8, 0.1 mmol$^{-1}$ FCCP, 0.5 mmol$^{-1}$ L-[^3H]leucine (80 $\mu$Ci ml$^{-1}$) and 0–60 mmol$^{-1}$ K$_2$SO$_4$. Each point represents the mean±s.e. of a typical experiment carried out in quadruplicate. When not given, s.e. bars were smaller than the symbols used.
question inhibition experiments were performed. The inhibition pattern exerted on leucine uptake by eight amino acids, besides leucine, from the cis side of the membrane, in the presence of $\Delta \psi$ and of a pH gradient, was very similar with and without external potassium (Fig. 8). Those amino acids, i.e. alanine, phenylalanine and histidine, that are known to compete for the same translocator in the presence of potassium (Hanozet et al. 1989) exerted a strong inhibition on the uptake of leucine (over 80% of the control value), both with and without the driver cation. No interaction occurred in the absence of potassium between the carrier and proline, glycine, lysine or aspartic acid, which are also not recognized in the presence of potassium. These results support the hypothesis of a single carrier. Further, it should be noted that the inhibition pattern reported here is in full agreement with those previously obtained in different experimental conditions (Giordana et al. 1989). A carrier-mediated leucine transport not influenced by glucose, glycine, arginine or glutamic acid was also found in the midgut of the honeybee in vivo (Crailsheim, 1988).

The results of counterflow experiments are reported in Fig. 9. The vesicles were preloaded with 10 mmol\textsuperscript{−1} cold leucine and the uptake of 0.5 mmol\textsuperscript{−1} labelled amino acid vs time was measured in the absence of potassium or in the presence of

![Fig. 7. Kinetics of potassium-independent leucine uptake as a function of cis leucine concentration in the presence of a pH gradient with and without FCCP. Eadie–Hofstee plot of the data after subtraction of a linear component. BBMV conditions at zero time. (○) in: 100 mmol\textsuperscript{−1} mannitol, 10 mmol\textsuperscript{−1} Hepes–Tris, pH 7.4; out: 100 mmol\textsuperscript{−1} mannitol, 10 mmol\textsuperscript{−1} Hepes–Tris, pH 7.4, 0.05–5 mmol\textsuperscript{−1} L-[^3H]leucine (80 µCi ml\textsuperscript{−1}). (■) in: 160 mmol\textsuperscript{−1} mannitol, 90 mmol\textsuperscript{−1} Hepes, 45 mmol\textsuperscript{−1} Tris, pH 7.4; out: 184 mmol\textsuperscript{−1} mannitol, 57 mmol\textsuperscript{−1} Tris, pH 8.8, 0.05–5 mmol\textsuperscript{−1} L-[^3H]leucine (80 µCi ml\textsuperscript{−1}), 0.1 mmol\textsuperscript{−1} FCCP. (▲) in: 160 mmol\textsuperscript{−1} mannitol, 90 mmol\textsuperscript{−1} Hepes, 45 mmol\textsuperscript{−1} Tris, pH 7.4; out: 184 mmol\textsuperscript{−1} mannitol, 57 mmol\textsuperscript{−1} Tris, pH 8.8, 0.05–5 mmol\textsuperscript{−1} L-[^3H]leucine (80 µCi ml\textsuperscript{−1}). Kinetic parameters were obtained by linear regression analysis according to the least-squares method. Each point represents the mean±s.e. of a typical experiment carried out in quadruplicate. When not given, s.e. bars were smaller than the symbols used.}
Table 4. The effect of pH gradient and FCCP on kinetic parameters of potassium-independent leucine uptake

<table>
<thead>
<tr>
<th>FCCP</th>
<th>ΔpH</th>
<th>$K_m$ (mmol⁻¹)</th>
<th>$V_{max}$ (pmol 7 s⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>0.40±0.02</td>
<td>848±62</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>0.36±0.02</td>
<td>1293±38</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.24±0.01</td>
<td>1565±41</td>
</tr>
</tbody>
</table>

Experimental condition as in Fig. 6. Means±s.e.

Table 5. Trans effect of leucine on leucine kinetic parameters in the absence of Δψ and potassium

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mmol⁻¹)</th>
<th>$V_{max}$ (pmol 7 s⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.430±0.02</td>
<td>777±87</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.330±0.09</td>
<td>1168±112</td>
</tr>
</tbody>
</table>

BBMV conditions at zero time. In: 100 mmol⁻¹ mannitol, 10 mmol⁻¹ Hepes-Tris, pH 7.4, without (control) or with 2 mmol⁻¹ leucine; out: 100 mmol⁻¹ mannitol, 10 mmol⁻¹ Hepes-Tris, pH 7.4, 0.1–9.6 mmol⁻¹ L-[³H]leucine (80 μCi ml⁻¹).

Kinetic parameters were obtained from the Eadie-Hofstee plot of the data by linear regression analysis according to the least-squares method. Mean±s.e. The experiment was performed in quadruplicate.

equal potassium concentrations inside and outside the vesicles (50 mmol⁻¹ K₂SO₄). The potassium ionophore valinomycin was added to obtain a short circuit via K⁺ permeability (Kessler and Toggenburger, 1979). The time courses obtained were extremely different: in the presence of potassium, the uptake was higher at very short incubation times and lower later in comparison with the behaviour observed in the absence of the driver cation.

Discussion

The data presented in this paper show that most leucine uptake in BBMV from Philosamia cynthia midgut takes place through a potassium-dependent cotransport mechanism, as observed for all the amino acids studied so far in lepidopteran enterocytes (Hanozet et al. 1980; Giordana et al. 1982, 1985; Sacchi et al. 1984; Wolfersberger et al. 1987). Leucine shares with most neutral amino acids a rather nonspecific transport system (Hanozet et al. 1989; Giordana et al. 1989; Fig. 8), which is strongly dependent on the transmembrane potential. In fact, the Δψ-dependence of leucine uptake is supported by three different lines of experimental
evidence: (1) the uptake value depends on the anions of the potassium salts used (Table 2) in a way presumably related to anion permeabilities and therefore to $\Delta \psi$ modulations; (2) experiments performed with the electrical potential difference short-circuited by the highly permeant anion NO$_3^-$ show a reduction of the uptake (Fig. 2); (3) the protonophore FCCP, which induces a proton diffusion potential in the presence of a pH gradient, causes a large increase in uptake (Table 4).

A pH gradient improves the kinetic properties of the transporter both in the presence and in the absence of potassium (Tables 3 and 4). Considering that in vitro leucine concentrations in the lumen, midgut cells and hemolymph of *Philosamia cynthia* larvae are 0.24, 0.60 and 0.58 mmol l$^{-1}$, respectively (Parenti *et al.* 1984), and that a steep pH gradient (3-4 pH units) is present at the luminal border of the epithelial cell (Dow, 1984), in principle this large gradient could be used to drive the amino acid uptake via a proton–amino acid antiport, as described for rat kidney by Stieger *et al.* (1983). However, experiments performed with vesicles prepared in a buffer at pH 7.4 and incubated in a solution at pH 8.9 showed no transient accumulation of leucine in the absence of potassium (data not shown), so protons do not appear to be directly involved in leucine uptake. In BBMV of lobster hepatopancreas, leucine uptake is stimulated by an acidic pH, which resembles the physiological pH of the lumen, and seems to be a sodium- and chloride-dependent electroneutral process (Ahearn and Clay, 1988).
Leucine uptake in BBMV from insect midgut

Fig. 9. Counterflow accumulation of leucine in the presence or in the absence of potassium. BBMV condition at zero time. In: 100 mmol\textsuperscript{−1} mannitol, 10 mmol\textsuperscript{−1} Hepes-Tris, pH 7.4, 10 mmol\textsuperscript{−1} leucine, with (▲) or without (●) 50 mmol\textsuperscript{−1} K\textsubscript{2}SO\textsubscript{4}. In the presence of potassium, BBMV were preincubated with valinomycin (2 μg mg\textsuperscript{−1} protein); out: 100 mmol\textsuperscript{−1} mannitol, 10 mmol\textsuperscript{−1} Hepes-Tris, pH 7.4, 0.5 mmol\textsuperscript{−1} L-[\textsuperscript{3}H]leucine (30 μCi ml\textsuperscript{−1}) with (▲) or without (●) 50 mmol\textsuperscript{−1} K\textsubscript{2}SO\textsubscript{4}. Each point represents the mean±s.e. of a typical experiment carried out in triplicate. When not given, s.e. bars were smaller than the symbols used.

Leucine uptake as a function of cis potassium concentration shows a hyperbolic relationship (Fig. 6), which suggests a 1:1 ratio between the driver cation and the cotransported amino acid although, as stated by Restrepo and Kimmich (1985), this is not conclusive proof of a 1:1 coupling stoichiometry of the transported species.

About 20% of the uptake of leucine measured at a saturating potassium concentration is potassium-independent (Fig. 6, inset) and this component is also carrier-mediated (Fig. 7). The similar inhibition patterns exerted by the tested amino acids on the initial uptake rate of leucine in the presence and in the absence of potassium strongly suggest the existence of a single carrier that can cross the membrane as a binary or ternary complex. The relative mobilities are: ternary complex>binary complex (carrier and leucine)>unloaded carrier. In fact, in the absence of potassium, the trans-stimulation exerted by leucine indicates that the binding of the amino acid at the trans side of the membrane has a stimulatory effect, possibly increasing the mobility of the carrier. An opposite trans effect of a cotransported nutrient was reported by Kessler and Semenza (1983), who observed in BBMV from rat intestine a trans inhibition of glucose uptake caused by a high intravesicular concentration of glucose. This trans effect was ascribed to the low mobility, if any, of the binary complex carrier and glucose. Further, the lower uptake values found with potassium in countertransport experiments would be explained by the higher mobility of the ternary complex, which would cause a more rapid dissipation of the gradients. The binary and ternary complexes are
affected in a similar way by a pH gradient, but only the ternary complex is sensitive to $\Delta \psi$.

References


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