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FUNCTIONAL CHARACTERIZATION OF LEUCINE TRANSPORT INDUCED IN *XENOPUS LAEVIS* OOCYTES INJECTED WITH mRNA ISOLATED FROM MIDGUTS OF LEPIDOPTERAN LARVAE (*PHILOSAMIA CYNTHIA*)

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Summary

The injection of poly(A)+ mRNA prepared from *Philosamia cynthia* midgut caused time- and dose-dependent increases of leucine transport in *Xenopus laevis* oocytes, with an increase in leucine uptake 1.5–3 times that of oocytes injected with water. When the NaCl concentration was reduced from 100 to 5 mmol l^{-1}, the difference between mRNA- and water-injected oocytes was greater and a fourfold increase of L-leucine uptake was measured. D-Leucine (10 mmol l^{-1}) completely inhibited the induced uptake of 0.1 mmol l^{-1} L-leucine. The newly expressed component of L-leucine uptake increased at alkaline pH and was abolished by incubation for 15 min with 15 mmol l^{-1} phenylglyoxal. The mean K_m values, calculated using Na^+ activation curves of leucine uptake, were 23.3±6.1 mmol l^{-1} in water-injected oocytes and 0.4±0.2 mmol l^{-1} for the newly expressed component of leucine uptake in mRNA-injected oocytes. On the basis of these results, we conclude that the increase of L-leucine uptake in mRNA-injected oocytes was due to the expression of a new transport system, which differs from the endogenous ones and shares many features with that found previously in *Philosamia cynthia* midgut.

Key words: leucine cotransport, expression, lepidopteran larva, midgut, *Philosamia cynthia*.

Introduction

The epithelial layer of the midgut of *Philosamia cynthia* larvae consists mainly of absorptive columnar cells and goblet cells. The latter contain a vacuolar-type proton pump that energizes net potassium secretion into the lumen (Harvey and Nedergaard, 1964; Wieczorek *et al.* 1991). The transmucosal electrical potential difference in columnar cells can easily reach 150 mV, with the positive pole in the lumen. This electrical potential difference is the driving force for a cotransport mechanism coupling amino acid and potassium fluxes *in vivo* (Giordana *et al.* 1982). The main feature of the cotransport mechanism is the lack of cation specificity. Either potassium or sodium can drive leucine uptake into brush-border membrane vesicles from *Philosamia cynthia* midguts. However, the affinity of the transporter for sodium is higher than that for potassium (Hanozet *et al.* 1992; Sacchi *et al.* 1994).

To obtain further information about this particular transport mechanism, which has been thoroughly studied in both isolated midguts and brush-border membrane vesicles, we have tried to express it in *Xenopus laevis* oocytes. The expression of a functional transport protein in oocytes alters the transport activity already present in oocytes or induces a new transport activity with features that closely resemble those of the tissues from which the mRNA has been purified. The expression is the first step of the functional expression cloning technique that can lead to the identification and sequencing of the cDNA from which the structure of proteins can be deduced. The primary structures of some amino acid transporters have been discovered in recent years with experimental procedures based on molecular biology (Pines *et al.* 1992; Well and Hediger, 1992; Kanai and Hediger, 1992; Markovich *et al.* 1993; Bertran *et al.* 1992, 1993; Shafqat *et al.* 1993; Tate *et al.* 1992; Magagnin *et al.* 1992).

In the present paper, we report time- and dose-dependent increases of leucine transport in *Xenopus laevis* oocytes injected with poly(A)+ RNA isolated from the midgut of a lepidopteran larva, *Philosamia cynthia.* We suggest that this increase is the result of the expression of a new transport system that shares many features of that found in *Philosamia cynthia* midgut.

Materials and methods

Oocyte injection

Small clumps of *Xenopus laevis* oocytes were treated with

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2 mg ml^{-1} collagenase in a Ca^{2+}-free solution (in mmol l^{-1}: 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 10 Hepes/Tris, pH 7.5) for 1 h and then with a fresh solution of collagenase for a further hour to remove the follicular layer. After thorough washing (composition of the washing buffer: 88 mmol l^{-1} NaCl, 1 mmol l^{-1} KCl, 0.82 mmol l^{-1} $MgCl_2$, 0.41 mmol l^{-1} $CaCl_2$, 0.33 mmol l^{-1} $Ca(NO_3)_2$, 20 μg ml^{-1} gentamycin, 10 mmol l^{-1} Hepes/Tris, pH 7.5), the oocytes were kept overnight in this solution at 18 °C. Healthy-looking stage V–VI oocytes were injected with quantities of mRNA ranging from 5 to 25 ng in 50 nl of water or with the same volume of water using a manual injector (Drummond). Unless otherwise stated, oocytes were incubated at 18 °C for 3–5 days before the uptake measurements.

Isolation of poly(A)+ RNA

Midgut total RNA from fifth-instar larvae was extracted using the phenol-extraction methods as described by Chomczynki and Sacchi (1987). Polyadenylated RNA [poly(A)+ RNA] was obtained by affinity purification on oligo (dT)–cellulose purchased from Sigma (Sambrook *et al.* 1989).

Leucine uptake measurements in oocytes

The uptake of L-[4,5-^{3}H]leucine, obtained from the Radiochemical Centre (Amersham International, Amersham, UK), was measured in 7–10 *Xenopus* oocytes per individual time point or condition. Oocytes were placed in 100 μl of uptake solution having the following composition (in mmol l^{-1}): 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes/Tris, pH 7.5, plus 100 μmol l^{-1} L-[4,5-^{3}H]leucine (1.48 MBq ml^{-1}). In experiments performed at low Na^+ concentration or while studying the kinetics of leucine transport as a function of Na^+ concentration, NaCl was replaced by an equivalent amount of choline chloride. Unless otherwise stated, uptake was for 40 min at room temperature, this being the shortest time that could be used to produce clearly detectable differences between experimental and control groups. After incubation, the oocytes were washed three times with 4 ml of the following solution (in mmol l^{-1}: 100 choline chloride, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes/Tris, pH 7.5). Single oocytes were transferred to a scintillation vial, dissolved in 0.2 ml of 10 % sodium dodecylsulphate (SDS) and counted for radioactivity in 5 ml of scintillation solution.

Preparation of brush-border membrane vesicles

Midguts from fifth-instar larvae of *Philosamia cynthia* (Saturnidae) were frozen and stored in liquid nitrogen (Giordana *et al.* 1992). Samples of the frozen midguts (2–3 g) were rapidly thawed at 37 °C and then used to prepare brush-border membrane vesicles (BBMVs) by calcium precipitation (Sacchi *et al.* 1990). BBMVs were resuspended at protein concentrations of 5–10 mg ml^{-1}, determined according to Bradford (1976).

Transport experiments in BBMVs

Transport experiments were performed in quadruplicate using a rapid filtration technique described previously (Sacchi *et al.* 1990). All the experiments were performed in the presence of a pH gradient, $7.4_{in}/8.9_{out}$, similar to that found *in vivo* across the mucosal brush border of the posterior midgut, and an electrical potential difference, negative inside the vesicles, obtained by adding the protonophore carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) to the incubation mixture (100 μmol l^{-1} final concentration). Osmolarity was kept constant with mannitol, so that no osmotic gradient existed across the membrane vesicles at time zero.

The composition of the solutions inside and outside the vesicles at time zero was as reported in the legend to Fig. 6. Kinetic features were determined in conditions of initial uptake rate (3 s). Data were analyzed by computer using a multiparameter, iterative, nonlinear regression program based on the Marquardt–Levemberg algorithm (Sigmaplot, Jandel, CA).

Results and discussion

The injection of whole poly(A)+ mRNA prepared from *Philosamia cynthia* midgut resulted in a 1.5- to threefold higher uptake of L-leucine than that found in oocytes injected with water. Fig. 1 shows the time courses of Na^+-dependent leucine uptake in oocytes 3 days after injection with 50 nl of water or with 25 ng of mRNA in the same volume of water. The difference between water- and mRNA-injected oocytes can be better observed in Fig. 2, which shows experiments

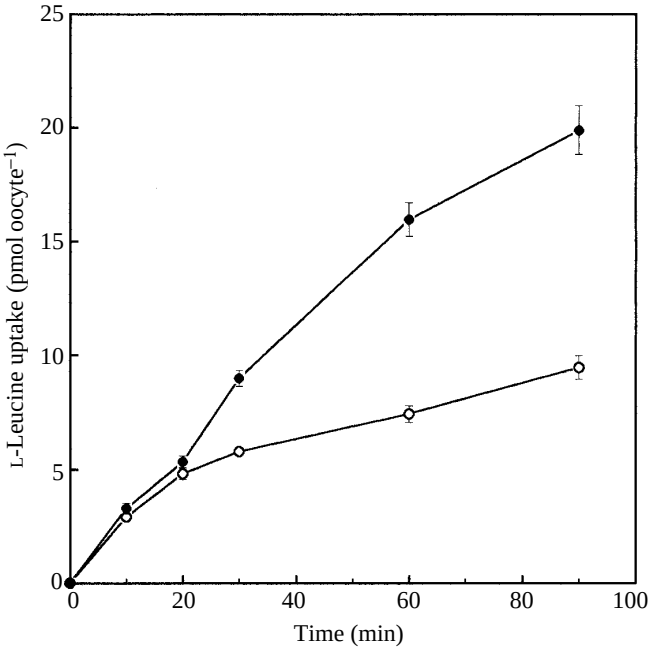

Fig. 1. Time courses of L-leucine uptake by water- and mRNA-injected *Xenopus* oocytes. The uptake of 0.1 mmol l^{-1} L-leucine was measured 3 days after the injection of 50 nl of water containing 0 ng (open circles) or 25 ng (filled circles) of mRNA. The uptake was measured after various periods of incubation in a solution containing 100 mmol l^{-1} NaCl. Values are means ± S.E.M. for seven oocytes in a representative experiment.

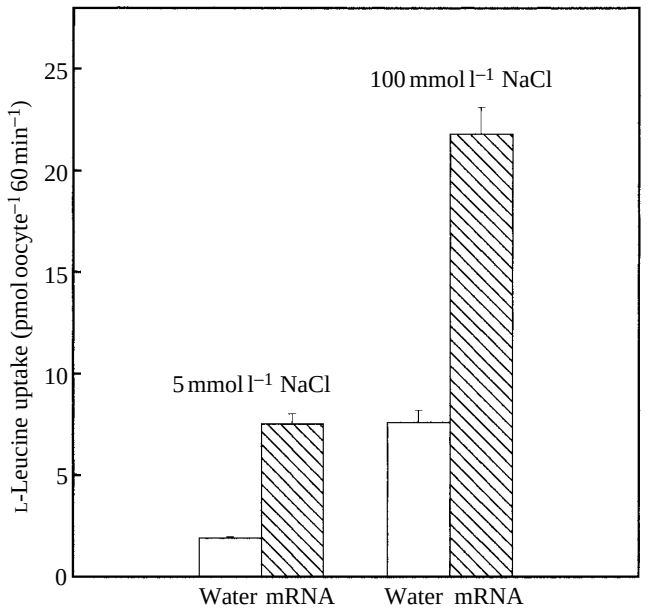

Fig. 2. Uptake of 0.1 mmol^{-1} L-leucine by *Xenopus* oocytes 5 days after the injection of 50 nl of water containing 0 ng (open columns) or 25 ng of mRNA (hatched columns) measured in solutions containing 5 and 100 mmol^{-1} NaCl. NaCl was replaced by choline chloride. Values are means + S.E.M. for seven oocytes in a representative experiment.

performed 5 days after the injection and carried out in the presence of 100 or 5 mmol l^{-1} NaCl. At 5 mmol l^{-1} NaCl, there was a fourfold increase of leucine uptake in oocytes injected with mRNA.

Fig. 3 shows the dose-dependent increase of leucine transport in oocytes injected with different amount of mRNA. Injections of mRNA exceeding 25 ng increased the mortality of the oocytes.

Since K^+ can drive amino acid transport in *Philosamia cynthia* midgut, the effects of K^+ on leucine uptake in control and mRNA-injected oocytes were investigated. No potassium activation could be demonstrated in the injected oocytes and so all succeeding experiments were performed with sodium or choline in the incubation solutions. It should be noted that in *Xenopus* oocytes the potassium gradient is outwardly directed, and when the external potassium concentration is increased, the electrical potential difference is drastically reduced (V. F. Sacchi, C. Perego and S. Magagnin, unpublished observations). Therefore, K^+ cannot drive L-leucine uptake because there is no inwardly directed electrochemical potential difference for K^+.

The characteristics of the induced leucine transport were investigated with a D amino acid that might be expected to interact with the intestinal leucine transporter. The resulting inhibitory effects of D-leucine on L-leucine uptake are shown in Fig. 4. D-Leucine is a potent inhibitor of the induced leucine transport. 10 mmol l^{-1} D-leucine inhibited total L-leucine uptake by 33%, which is a 75% inhibition of the induced leucine transport, whereas it inhibited the endogenous leucine uptake by only 5%. These results are in keeping with the data reported in BBMVs from *Philosamia cynthia* midgut, where leucine and

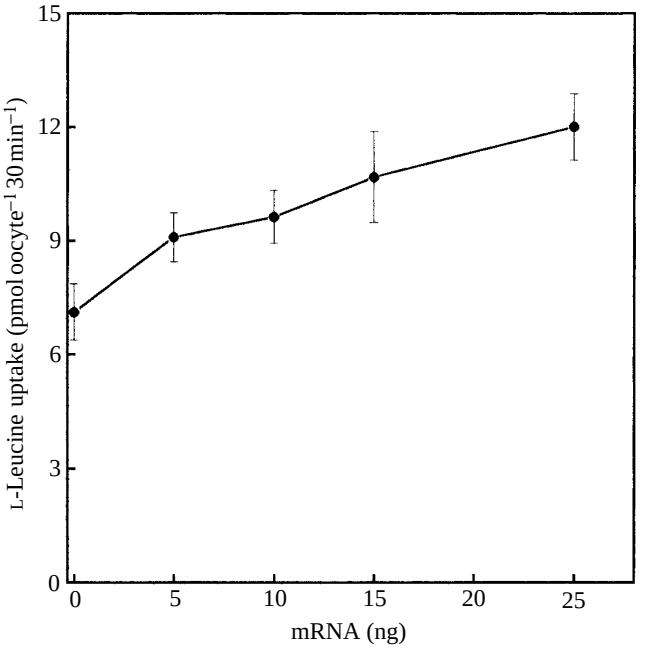

Fig. 3. Dose-dependent expression of L-leucine uptake. Water (50 nl) containing different amounts of mRNA was injected into *Xenopus* oocytes and 0.1 mmol l^{-1} L-leucine uptake was measured after 3 days in a solution containing 100 mmol l^{-1} NaCl. Values are means ± S.E.M. for seven oocytes in a representative experiment.

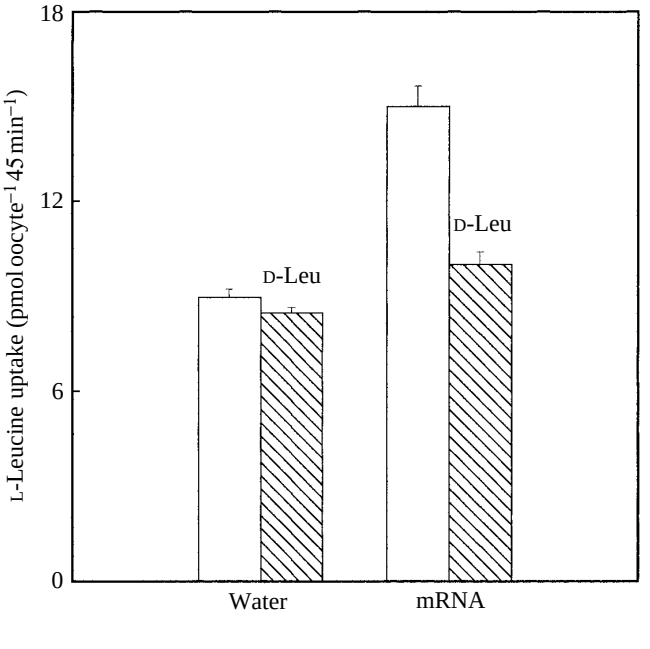

Fig. 4. Uptake of 0.1 mmol^{-1} L-leucine in water- and mRNA-injected *Xenopus* oocytes. The uptake was measured in a 5 mmol^{-1} NaCl solution in the absence (open columns) or in the presence (hatched columns) of 10 mmol l^{-1} D-leucine. Values are means + S.E.M. for seven oocytes in a representative experiment.

Table 1. *Influence of pH on Na^+-dependent L-leucine uptake in control and mRNA-injected oocytes*

	L-Leucine uptake (pmol oocyte^{-1} 15 min^{-1})		
pH	Control	mRNA	mRNA–control
6.0	3.90±0.10	5.47±0.14†	1.57
7.5	3.90±0.08*	6.20±0.23†	2.30
9.0	3.08±0.20*	6.20±0.29	3.12

0.1 mmol l^{-1} L-leucine uptake was measured in mRNA- and water-injected oocytes 4 days after injection. L-Leucine uptake was measured in the presence of 5 mmol l^{-1} NaCl.

Data are means ± S.E.M. of 6-10 oocytes in a representative experiment.

All control values are significantly different from mRNA values (P<0.05). * and † indicate significant differences between means (P<0.05).

alanine are transported by the same system and each isomer inhibits the uptake of the other (Hanozet *et al.* 1984).

The intestinal fluid in the lumen of lepidopteran larvae has a very high pH (Dow, 1992) and, in agreement with this physiological property, the activity of the neutral amino acid transporter was maximal at alkaline pH values (Sacchi *et al.* 1990; Hennigan *et al.* 1993). The effect of pH on the induced Na^+-dependent L-leucine transport was investigated by measuring L-leucine uptake at three different pH values: 6, 7.5 and 9. Table 1 reports the effect of pH on L-leucine uptake in

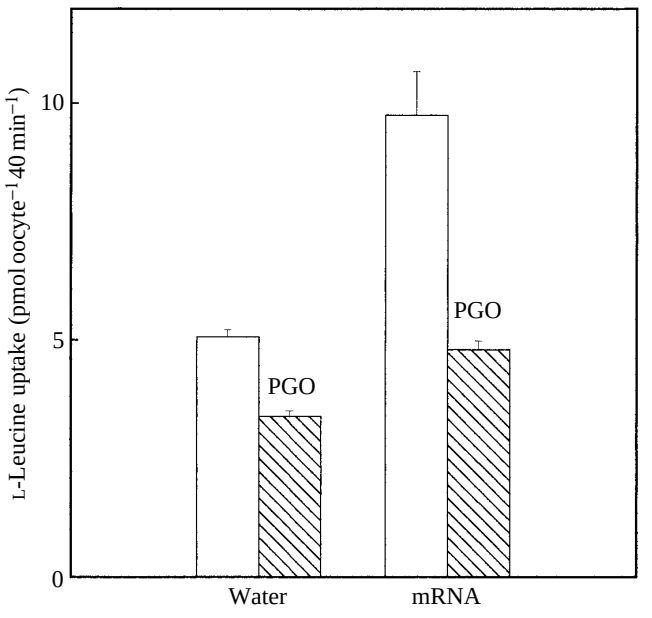

Fig. 5. Effect of phenylglyoxal (PGO) on the uptake of 0.1 mmol l^{-1} L-leucine in water- and mRNA-injected *Xenopus* oocytes at 5 mmol l^{-1} NaCl. Oocytes were incubated for 15 min in a solution containing 0 mmol l^{-1} (open columns) or 15 mmol l^{-1} (hatched columns) PGO. Values are means + S.E.M. for 6–10 oocytes in a representative experiment.

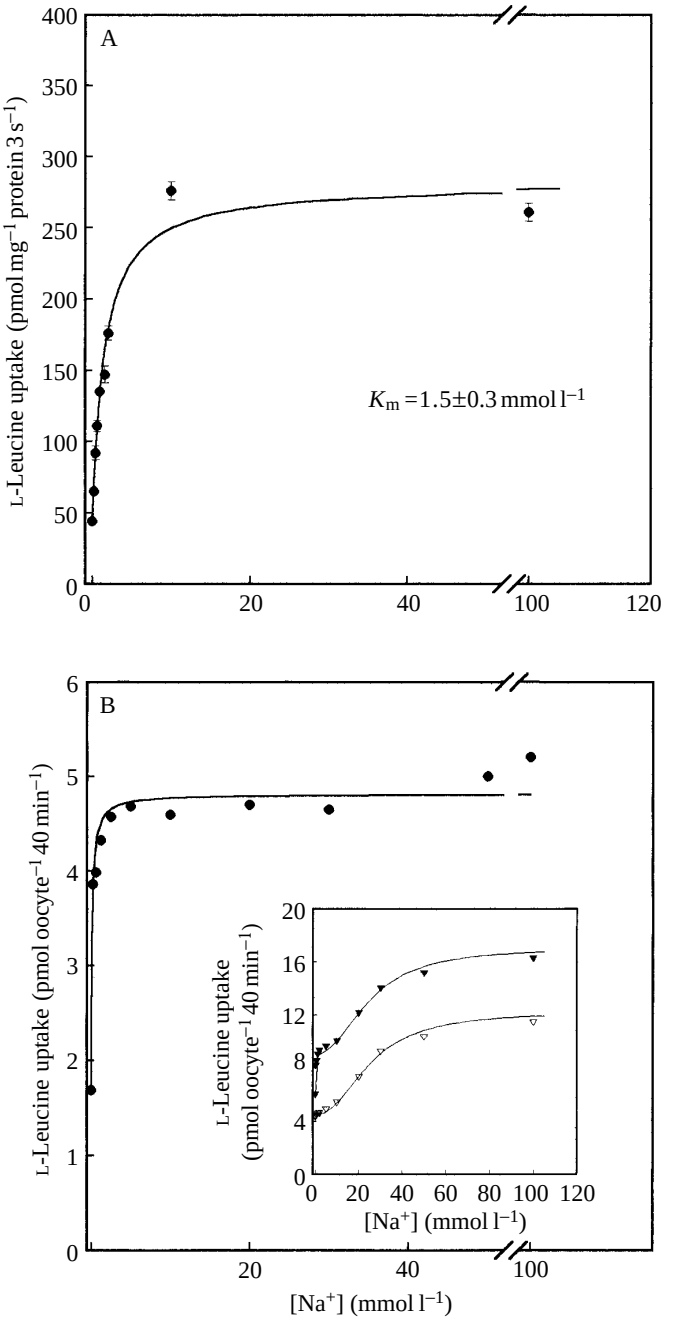

Fig. 6. (A) Uptake of 0.1 mmol l^{-1} L-leucine as a function of Na^+ concentration in brush-border membrane vesicles. Vesicle internal solution contained (mmol l^{-1}): 225 mannitol, 90 Hepes, 45 Tris at pH 7.4. The vesicles were diluted 1:5 in a solution of the following final composition (in mmol l^{-1}): 85 mannitol, 18 Hepes, 57 Tris, pH 8.9, and 0–100 NaCl plus 200–0 mannitol, 0.1 FCCP and 0.1 L-[^{3}H]leucine (approximately 1.48 MBq ml^{-1}). Each point is the mean ± S.E.M. of a typical experiment performed in quadruplicate. (B) Net mRNA-induced uptake in *Xenopus laevis* oocytes of 0.1 mmol l^{-1} L-leucine as a function of Na^+ concentration. Uptake values are the differences between the curves shown in the inset. Inset: 0.1 mmol l^{-1} L-leucine uptake as a function of Na^+ concentration in water-injected (open triangles) and mRNA-injected (filled triangles) oocytes. NaCl was replaced by choline chloride. Values are means for 10 oocytes in a representative experiment.

control oocytes and in mRNA-injected oocytes in the presence of 5 mmol l^{-1} NaCl. Since an alkaline or acid pH could damage the oocytes, the uptake was measured after 15 min of incubation instead of after 40 min. Even under these conditions, it was apparent that the net L-leucine transport induced by mRNA injection increased at alkaline pH. These results are consistent with the expression of a neutral amino acid transport system which is present on the brush-border membrane of *Philosamia cynthia* enterocytes and is known to be activated by alkaline pH values (Sacchi *et al.* 1990). Similar results were also obtained in the presence of 100 mmol l^{-1} NaCl (not shown).

The effect of phenylglyoxal (PGO), a reagent specific for arginine residues, on L-leucine uptake in water- and mRNA-injected oocytes is shown in Fig. 5. Incubation of oocytes for 15 min with 15 mmol l^{-1} PGO inhibited L-leucine uptake in both control and experimental oocytes, but the uptake was 33 % inhibited in controls and 50 % inhibited in mRNA-injected oocytes. In other words, the newly expressed component of L-leucine uptake in mRNA-injected oocytes was more sensitive to PGO than was L-leucine uptake in control oocytes. This effect is in agreement with the results reported by Parenti *et al.* (1994), who found that PGO strongly inhibited L-leucine uptake in BBMVs from *Philosamia cynthia* midgut and suggested that arginine residues play an important role in the translocation step of L-leucine in this tissue.

Since the greatest difference between water- and mRNA-injected oocytes was observed at 5 mmol l^{-1} Na^+, experiments were performed to estimate the affinity for Na^+ of the induced L-leucine transport. Sodium activation curves of leucine uptake in BBMVs from *Philosamia cynthia* midgut and *Xenopus laevis* oocytes are shown in Fig. 6A,B. Fig. 6A shows 0.1 mmol l^{-1} leucine uptake in BBMVs purified from the midgut of *Philosamia cynthia* as a function of sodium concentration. The K_m for sodium is 1.5 mmol l^{-1}, a value considerably lower than those of Na^+-dependent cotransporters in other species. This high affinity for sodium of the binding site on the transporter was previously investigated in brush-border membrane vesicles from *Philosamia cynthia* and it can be explained by the very low sodium concentration (1 mmol l^{-1}) in the midgut lumen (Hanozet *et al.* 1992; Sacchi *et al.* 1994). The inset in Fig. 6B shows sodium activation curves in water- and mRNA-injected oocytes. Note that the curve was sigmoidal in control oocytes. The difference between the two curves (Fig. 6B) should give the sodium activation of the newly expressed leucine transport. The mean K_m value calculated from three independent measurements of the kinetics of the process was 0.4±0.2 mmol l^{-1}, an order of magnitude lower than the mean K_m value (23.3±6.1 mmol l^{-1}, mean ± S.E.M. of three independent measurements of the uptake kinetics) found in control oocytes.

The presence of an endogenous Na^+-dependent L-leucine uptake in oocytes makes it more difficult to analyze the induced L-leucine transport. However, the kinetic features of the endogenous and induced transporters are so different that it is possible to distinguish between them. These kinetic features explain why the greatest difference between controls and mRNA-injected oocytes was observed at 5 mmol l^{-1} Na^+ (Fig. 2). Under these experimental conditions, the induced Na^+-dependent L-leucine uptake was almost completely activated because the transport system has a very high affinity for sodium, whereas the endogenous Na^+-dependent transport system was only partially activated, both because the affinity for sodium is much lower and because the activation curve is sigmoidal.

In studies expressing foreign mRNA in oocytes, it is not possible to exclude the activation of the large amount of endogenous mRNA or endogenous transporters, but circumstantial evidence suggests that in the present study the increase of L-leucine uptake in injected oocytes was due to the expression of a new transport system that differed from the endogenous transporters and had many features found previously in the amino acid transport system in *Philosamia cynthia* midgut.

The experiments presented in this work confirm that mRNA purified from midgut cells of Lepidoptera can be expressed in oocytes and provide a basis for expression cloning of the protein that effects neutral amino acid transport in the enterocytes of *Philosamia cynthia* larvae.

Lepidoptera are considered to be a highly specialized order of insects, and the features of the cation-dependent amino acid cotransport may have evolved from a more primitive sodium-dependent mechanism. Comparison of known H^+ or Na^+ cotransporters with the primary structure of this transporter might shed light on the molecular evolution of these transport systems.

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