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ANOMALOUS GLUTAMATE/ALKALI CATION SYMPORT IN LARVAL *MANDUCA SEXTA* MIDGUT

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Summary

Rapid filtration assays were used to characterize glutamate/cation uptake in brush-border membrane vesicles from the larval midgut of the lepidopteran *Manduca sexta*. At pH 10.5, which is close to the physiological pH in the midgut of *M. sexta*, an inwardly directed K^+ gradient stimulated glutamate uptake, suggesting that glutamate was symported. Gradients of Na^+ or Li^+ were less effective. Neither Rb^+ nor Cs^+ stimulated glutamate uptake. Anion-specificity was less pronounced: the accumulation maximum was only slightly higher with thiocyanate (SCN^-) than with Cl^-, although initial uptake was noticeably faster with thiocyanate.

A distinct set of amino acids that would *cis*-inhibit or *trans*-elicit glutamate uptake was not found. Even L-glutamate itself did not elicit accumulations of labeled glutamate. Taken together, these results suggest that a glutamate-specific symporter may not be present. Moreover, because glutamate symport was found to be electroneutral *in vitro* whereas amino acid uptake is electrophoretic *in vivo*, we infer that symport with K^+ may not be an important mechanism of glutamate translocation by *M. sexta* midgut.

Introduction

Glutamate uptake in lepidopteran midgut (Wolfersberger *et al.* 1987; Giordana *et al.* 1989; Hanozet *et al.* 1989; Wolfersberger, 1993) presents a paradox. This anionic amino acid links the Krebs and urea cycles and hence is produced continuously in all cells. In the midgut of *Manduca sexta*, a 5000-fold electrical gradient favors the exit of glutamate from the cell to the lumen. For the amino acid to be driven *into* cells against this powerful force, a symport cycle would have to be electrophoretic and transfer net positive charge. Nevertheless, experimental data suggest that glutamate transport is electroneutral in lepidopteran (Giordana *et al.* 1989) and mammalian (Schneider *et al.* 1980; Corcelli *et al.* 1982) tissues. Glutamate symport in lepidopteran midgut, therefore, must either be extremely unusual or, in the absence of selective pressure for the evolution of a glutamate-specific symporter ('glutamate symporter'), not exist at any physiologically relevant level.

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M. sexta, an intensively studied lepidopteran, provides a tractable experimental model with which to resolve this paradox. The 1000-fold gain in body mass between egg and fifth-instar larva requires a substantial flux of amino acids to be used as an energy source (Parenti *et al.* 1985) and for growth. Not surprisingly, studies on larval *Philosamia cynthia* midgut brush-border membrane vesicles (BBMV) have identified six putative symporter families, including one each for acidic, neutral and basic amino acid groups (Giordana *et al.* 1989).

Earlier studies on amino acid symport in lepidopteran larvae have been conducted at near-neutral pH values (e.g. Giordana *et al.* 1982; Sacchi *et al.* 1984, 1990). Recently, however, Parenti *et al.* (1992) characterized leucine uptake at pH 8.8 in larval *Philosamia cynthia* midgut BBMV. Hennigan *et al.* (1993*a,b*) identified a system-B-type symporter in larval *M. sexta* midgut BBMV that symports zwitterionic amino acids optimally at pH 10, which is close to the average pH (10.5) found in the midgut contents (Dow, 1984). K^+ is the preferred cation for the symport of both neutral and basic amino acids, especially at pH 10 (Hennigan *et al.* 1993*b*). This finding is not altogether unexpected, since K^+ is the predominant cation in the lumen (Dow *et al.* 1984) and is required for transepithelial transport of amino acids (Nedergaard, 1972).

Amino acid uptake by lepidopteran midgut differs from uptake in more familiar crustacean and vertebrate tissues in four ways. (1) Uptake is energized by an H^+-translocating V-ATPase coupled tightly to a $K^+/2H^+$ antiporter; the resulting potassium electrochemical potential gradient is transformed into a large potential difference (PD) that drives uptake across the apical membrane. (2) K^+ and not Na^+ is the predominant cation. (3) CO_3^{2-} is the dominant anion, the concentration of Cl^- being small, so that the lumen is quite alkaline. The extracellular side is close to neutral pH. In contrast, crustacean and vertebrate membranes are energized by ion gradients secondary to K^+/Na^+-ATPase activity and Na^+ is the major extracellular cation and Cl^- the major anion. In short, amino acid/K^+ uptake in lepidopteran midgut is driven electrophoretically by an electrical PD, whereas amino acid/Na^+/Cl^- uptake in crustacean and vertebrate tissues is driven by an ionic gradient. Inasmuch as glutamate was found to be an extremely poor substrate for the neutral/basic amino acid symporter (Hennigan *et al.* 1993*a*) or any other symporter characterized to date in *M. sexta*, an evaluation of glutamate uptake under alkaline conditions seemed important.

Materials and methods

Second- and third-day, fifth-instar *M. sexta* larvae, weighing 5.2±0.6 g, were reared from eggs under constant light at 27 °C on an artifical diet (Carolina Biological Supply Company, Burlington, NC).

BBMV were isolated from midguts following the methods of Biber *et al.* (1981), as modified by Wolfersberger *et al.* (1987). Estimates of BBM purity *via* marker enzyme assays were reported by Eisen *et al.* (1989). The vesicles were loaded with resuspension buffer by equilibration for 1 h on ice, followed by centrifugation for 30 min at 31 000 ***g***. The method of Bradford (1976), using a commercial dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as standard, was used to determine protein concentrations.

Uptake was measured using published protocols (Hanozet *et al.* 1980), as modified by Wolfersberger *et al.* (1987) and Hennigan *et al.* (1993*a,b*). The vesicles were loaded with resuspension buffer [100 mmol l^{-1} mannitol, 50 mmol l^{-1} aminomethylpropanediol (AMPD) acidified with HCl to pH 10] for experiments with a salt gradient. For experiments without a salt gradient, the resuspension buffer also contained 100 mmol l^{-1} KSCN and the vesicles were treated with 8 μg valinomycin per mg protein to preclude the development of a cation gradient and the associated diffusion potential. 10 μl samples of the vesicle suspension were incubated with an equal volume of transport buffer (compositions indicated in the figure legends) for the desired interval and transport was quenched by the rapid addition of 4 ml of stop buffer. The diluted reaction mixture was filtered through a nitrocellulose filter supported on a sintered glass funnel. In countertransport experiments, the amino acid used as elicitor was added at a concentration of 20 mmol l^{-1} to the resuspension buffer. The transport buffer contained 50 mmol l^{-1} AMPD chloride at pH 10, 100 mmol l^{-1} mannitol and 100 mmol l^{-1} KSCN, together with 1 mmol l^{-1} labeled glutamate. The stop buffer had the same composition except that the amino acid was eliminated. In these experiments, 5 μl of the vesicle suspension were diluted into 95 μl of transport buffer and the incubation was allowed to proceed for the desired interval.

Experiments were conducted in triplicate on three separate preparations of vesicles and data were averaged over the three preparations; means ± S.E.M. are reported. Statistical analyses were performed using Student's *t*-test. A probability of 97.5 % was considered to represent a statistically significant difference.

Labeled glutamate (L-[U-^{3}H]glutamic acid) was purchased from Amersham (Arlington Heights, IL) and unlabeled amino acids were obtained from Sigma (St Louis, MO). The glutamate esters were obtained from Fluka (Ronkonkoma, NY). Other chemicals were obtained from Fisher (Pittsburgh, PA).

Results

Glutamate is taken up by BBMV

Preliminary experiments with a K^+ gradient showed that maximum L-glutamate uptake always occurred between 1.5 and 5 min of incubation and that equilibrium distribution of glutamate between the intravesicular and the extravesicular volumes was always reached within 60 min. Therefore, we chose sampling intervals (t) of 30 s, 60 s, 90 s, 2 min, 5 min, 10 min and 60 min in all subsequent experiments. The amount of non-specifically bound glutamate, approximated by the (extrapolated) t=0 uptake, was 0.3± 0.1 nmol mg^{-1} protein (see Fig. 6). Non-specific substrate binding contributes less than 5 % of the peak accumulation and 12 % of the equilibrium values, and the small correction was ignored. The time-dependence of glutamate uptake was insensitive to osmotic boundary conditions, as shown previously for leucine uptake (Hennigan *et al.* 1993*b*). Thus, a similar time course was obtained irrespective of whether iso-osmoticity was present at t=0 or t=∞ (Fig. 1). All of the work reported here was performed with iso-osmotic conditions present at t=∞. A K^+ gradient promoted the development of a transient maximum in the intravesicular concentration of labeled glutamate (Figs 1 and 2). The maximum accumulation, as well as the initial glutamate uptake rate (Fig. 2, inset), was

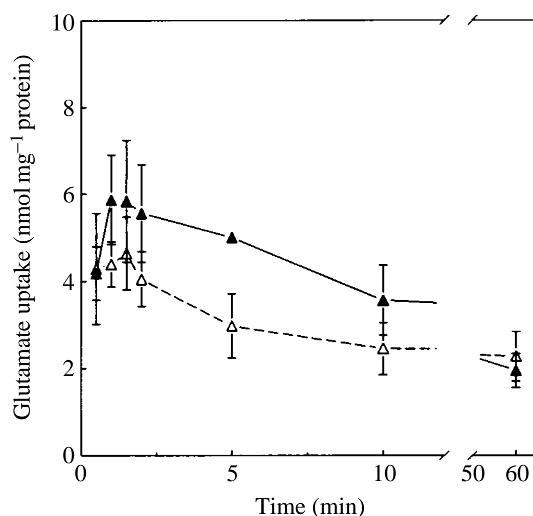

Fig. 1. Time course of uptake of glutamate under different osmolarity-balance conditions. To establish iso-osmolarity at *t*=60 min (▲), vesicles resuspended in 50 mmol l^{-1} AMPD and 100 mmol l^{-1} mannitol at pH 10 were mixed with samples of transport buffer (50 mmol l^{-1} AMPD, 100 mmol l^{-1}, (120 mmol l^{-1}) KSCN and 1 mmol l^{-1} [^{3}H]glutamate). Initial iso-osmolarity (at *t*=0, △) was achieved through the following changes: the resuspension buffer contained 200 mmol l^{-1} mannitol and the transport buffer contained 120 mmol l^{-1} KSCN and 0 mmol l^{-1} mannitol. When not given, the S.E.M. bars were smaller than the symbols used.

somewhat higher with SCN^- than with Cl^- as the counterion (Fig. 2A). The addition of valinomycin, a K^+-specific ionophore that dissipates the cation gradient, abolished the maximum (Fig. 2B). A choline gradient did not promote glutamate accumulations over those at equilibrium (Fig. 2B).

Glutamate uptake is stimulated by K^+

The effect of the alkali ions on glutamate uptake was examined at pH values of 8.9, 9.5 and 10.5, chosen to bracket the pK‴ of glutamate (9.67). The highest pH investigated approaches the *in vivo* luminal pH. At pH 8.9 (Fig. 3A) the uptake of glutamate was stimulated by initial gradients of K^+, Na^+ and Li^+. The maximum accumulations as a function of cotransported cation followed the sequence $K^+ \approx Na^+ \gg Li^+$, with the difference between K^+/Na^+ and Li^+ being statistically significant at a level of 97.5 % (Fig. 4).

At pH 9.7 (Fig. 3B) there is little change from the situation at pH 8.9, except for a marked decrease in the accumulation in the presence of Li^+ that is not significantly greater than 1. The difference between the effects of K^+ and Na^+ is again not statistically significant at the 97.5 % level (Fig. 4).

At pH 10.5 (Fig. 3C) the relative accumulations of glutamate in the presence of both K^+ and Na^+ were lower than at pH 9.7 or 8.9, but the maximum accumulation in the presence of K^+ was now significantly greater than that in the presence of Na^+ (97.5 % level). Li^+ does not stimulate glutamate uptake at pH 10.5 (Fig. 4). Neither Rb^+ nor Cs^+ stimulated glutamate uptake at any of the pH values tested. The equilibrium (60 min)

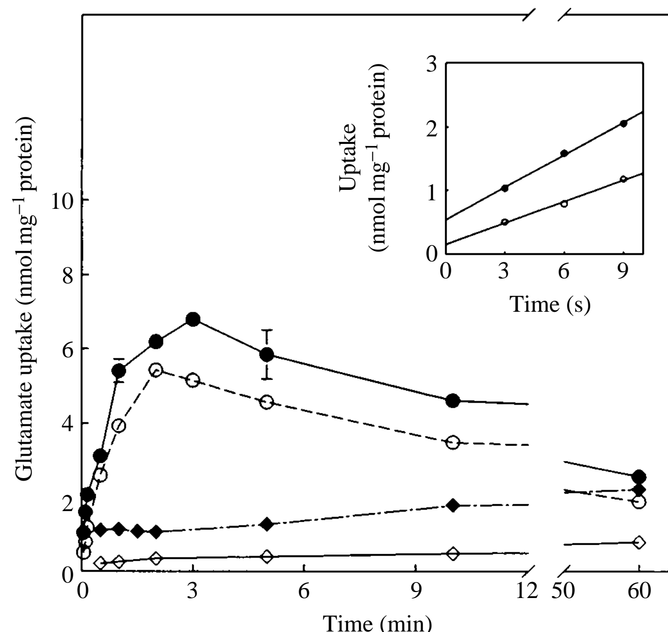

Fig. 2. Time course of glutamate uptake under different ionic gradient conditions. Vesicles resuspended in 100 mmol l^{-1} mannitol and 20 mmol l^{-1} cyclohexylaminoethane sulfonic acid (CHES)–Tris, at pH 9, were incubated with an equal volume of transport buffer containing 100 mmol l^{-1} mannitol, 20 mmol l^{-1} CHES-Tris, 1 mmol l^{-1} [^{3}H]glutamate and 100 mmol l^{-1} KCl (○) or 100 mmol l^{-1} KSCN (●), at pH 9. Inset: initial uptake rate of glutamate in the presence of 100 mmol l^{-1} KCl (○) or 100 mmol l^{-1} KSCN (●). Data from a single experiment conducted in triplicate, with the error bars denoting the S.E.M.; a replicate experiment yielded the same results. Valinomycin-treated vesicles (100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD, pH 10) were incubated with transport buffer of the same composition containing, in addition, 100 mmol l^{-1} KSCN (◆) and 1 mmol l^{-1} labeled glutamate. The same conditions were used (with untreated vesicles), except that 100 mmol l^{-1} choline chloride (◇) was used in place of KSCN.

glutamate concentration in all incubation mixtures shown in Fig. 3 was 2.60± 0.21 nmol mg^{-1} protein.

Glutamate uptake is electroneutral

The uptake of glutamate, leucine and lysine was investigated at three different membrane potentials. A proton diffusion potential was generated using a transmembrane pH imbalance in the presence of a protonophore, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). When the permeability of a given ion species (H^+ in this case) far exceeds that of the other ions, the Goldman–Hodgkin–Katz diffusion equation reduces to the Nernst–Einstein equilibrium equation. A pH difference of 1 unit then corresponds to a limiting potential difference (PD) of 59 mV at 25 °C. A K^+ gradient in the presence of valinomycin could not be used to generate a PD since K^+, the physiologically relevant anion, was the object of study as the symported cation.

The initial rates for glutamate uptake were found to be constant within experimental

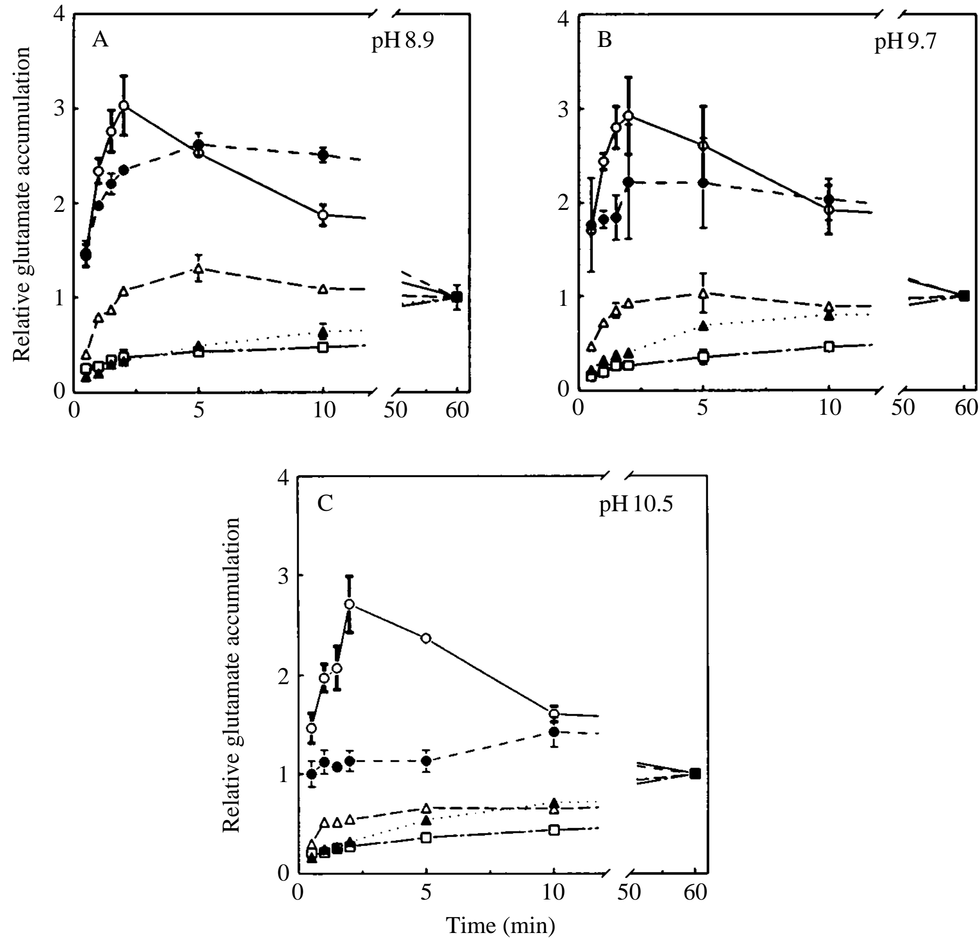

Fig. 3. Time course of glutamate uptake at the pH values indicated. Vesicles resuspended in $100\,mmol\,l^{-1}$ mannitol and $50\,mmol\,l^{-1}$ AMPD were mixed with samples of transport buffer at the same pH containing: $100\,mmol\,l^{-1}$ mannitol, $50\,mmol\,l^{-1}$ AMPD, $100\,mmol\,l^{-1}$ KCl (○), NaCl (●), LiCl (△), RbCl (▲) or CsCl (□) and $1\,mmol\,l^{-1}$ [^{3}H]glutamate. Relative accumulations, with their standard errors for triplicate BBMV preparations, were determined by dividing uptake at the sampling time by equilibrium uptake. When not given, the standard error bars were smaller than the symbols used.

error at intravesicular PD values of +59, 0 and −59 mV (relative to the extravesicular medium) (Fig. 5). By contrast, the initial rates of leucine and lysine uptake, measured as positive controls, were dependent upon intravesicular PD (Fig. 5).

Does the glutamate uptake agency have preferred substrates?

Cis-*inhibition experiments*

Initial rates of glutamate uptake with several inhibiting amino acids were tested in the presence and absence of a K^+ gradient. Uptake in the absence of inhibitor was used as a

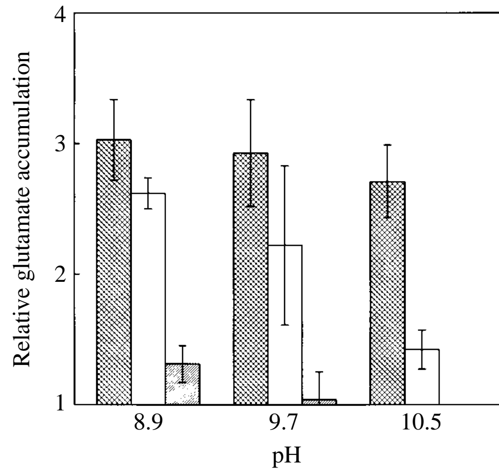

Fig. 4. Dependence of maximum glutamate uptake on alkali ion. Maximum relative accumulations shown for K^+ (cross-hatched bars), Na^+ (open bars) and Li^+ (hatched bars) were obtained from Fig. 3. Bars show ±S.E.M., *N*=9.

control in each case. Several likely inhibitors were used to demonstrate that the initial time courses were linear and that they had a common intercept, with a variation of less than 10 % (Fig. 6). The relative inhibitions, 1−(rate with inhibitor/rate without inhibitor),

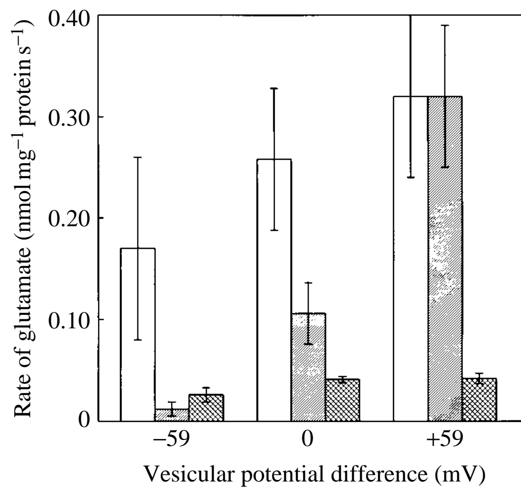

Fig. 5. Dependence of uptake rate in the presence of FCCP minus the uptake rate in the absence of FCCP on intravesicular potential difference. Vesicles resuspended in 100 mmol l^{-1} mannitol, 100 mmol l^{-1} KCl and 50 mmol l^{-1} AMPD at pH 9 were incubated with carbonylcyanide *p*-trifluoromethoxy phenylhydrazone (FCCP). Samples of the vesicle suspension were mixed with equal volumes of transport buffer at pH 8, 9 or 10, to yield limiting potential differences of +59, 0 and −59 mV, respectively, with respect to the extravesicular medium. The transport buffers contained 100 mmol l^{-1} mannitol, 100 mmol l^{-1} KCl, 50 mmol l^{-1} AMPD and 1 mmol l^{-1} [^{3}H]leucine (open bars), [^{3}H]lysine (hatched bars) or [^{3}H]glutamate (cross-hatched bars). Bars show ±S.E.M., *N*=9.

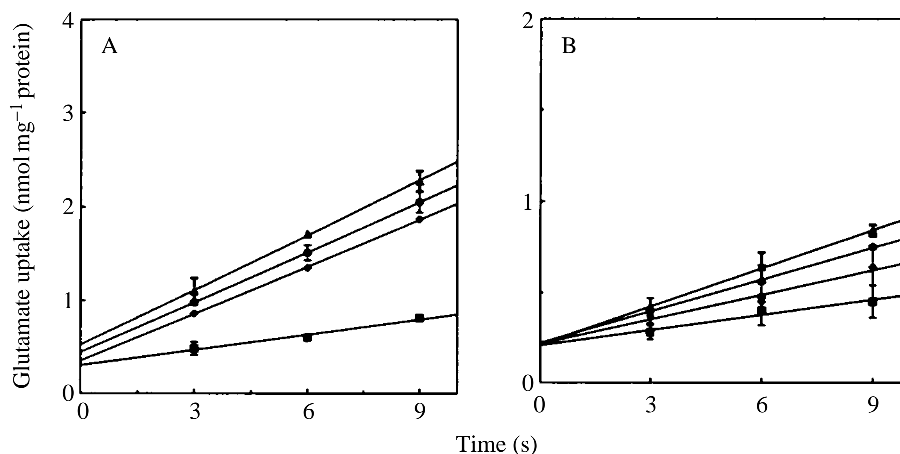

Fig. 6. Initial glutamate uptake over time. (A) BBMV in 100 mmol l^{-1} mannitol and 50 mmol l^{-1} AMPD at pH 10, were resuspended to give a final reaction mixture containing: 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD, 50 mmol l^{-1} KSCN, 0.5 mmol l^{-1} [^{3}H]-L-glutamate without (▲) or with 10 mmol l^{-1} lysine (●), cystine (◆), or glutamate (■) at pH 10. (B) KSCN was added to the resuspension buffer and the vesicles were treated with valinomycin. Other conditions as in A. When not given, the S.E.M. bars were smaller than the symbols used, N=9.

were then calculated, with the uptake at a single point in time substituted for the rate. The inhibition of uptake of radiolabeled glutamate in the presence and in the absence of a KSCN gradient by the 20 commonly occurring amino acids, D-glutamate, L-glutamic acid 5-methyl ester (5-MG) and L-glutamic acid dimethyl ester (DMG) is shown in Table 1. The amino acids are listed in their rank order of ability to inhibit K^+-gradient-driven uptake of labeled glutamate. Among the 23 amino acids tested, tryptophan, aspartate, glutamate and phenylalanine inhibited glutamate uptake most strongly.

To eliminate the possibility that symport of the ‘inhibiting’ amino acid diminishes the K^+ gradient, *cis*-inhibition experiments in the absence of a salt gradient were carried out (Table 1). The uptakes were reduced to as much as one-third of their ‘with-gradient’ values and their range was reduced sharply. In the absence of a K^+ gradient, phenylalanine inhibited the most (relative inhibition of 50 %) whereas leucine, tryptophan, serine, isoleucine, proline, lysine and 5-MG inhibited the least. There was no apparent difference among the remaining amino acids, with initial rates of glutamate uptake ranging from 0.37±0.03 to 0.48±0.08 nmol mg^{-1} protein 6 s^{-1}.

Countertransport experiments

The relative accumulation of labeled glutamate at time t', was calculated as uptake$_{t=t'}$/uptake$_{t=\text{equilibrium}}$. A study of the relative accumulation of labeled glutamate elicited by intravesicular glutamate (Fig. 7) revealed that maximum accumulation occurred around 90 s. Increasing the intravesicular glutamate concentration by a factor of 2 had no effect on the time course of uptake. t' was accordingly chosen to be 90 s in the

Table 1. *Effects of excess amounts of common amino acids on* L*-glutamate uptake in the presence and absence of a* K^+ *gradient*

Inhibitory amino acid	Uptake with gradient	Uptake without gradient
Trytophan	0.48±0.03	0.60±0.08*
Aspartate	0.57±0.06	0.40±0.02
Glutamate	0.60±0.04	0.40±0.08
Phenylalanine	0.62±0.04	0.32±0.05
Serine	0.91±0.08	0.51±0.10*
Alanine	1.06±0.05	0.38±0.06
Methionine	1.12±0.12	0.37±0.03
Leucine	1.15±0.01	0.56±0.16*
Isoleucine	1.15±0.10	0.56±0.16*
Glutamine	1.15±0.03	0.40±0.02
Histidine	1.18±0.03	0.39±0.05
Arginine	1.18±0.12	0.42±0.04
Threonine	1.22±0.05	0.42±0.04
Valine	1.24±0.07	0.48±0.08
Glycine	1.24±0.10	0.42±0.02
Tyrosine	1.26±0.09	0.47±0.04
Cystine	1.35±0.04	0.45±0.04
Aspargine	1.38±0.02	0.45±0.02
Proline	1.39±0.19	0.57±0.10*
Lysine	1.50±0.08*	0.65±0.18*
D-Glutamate	1.33±0.04	0.42±0.04
Dimethylglutamate	1.33±0.04	0.41±0.04
Glutamate 5-methyl ester	1.61±0.05*	0.50±0.11*
Control	1.71±0.02	0.64±0.08

Inhibition in the presence of a K^+ gradient: BBMV in 100 mmol l^{-1} mannitol and 50 mmol l^{-1} AMPD at pH 10, were resuspended to give a final reaction mixture containing 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD, 50 mmol l^{-1} KSCN, 10 mmol l^{-1} inhibiting amino acid and 0.5 mmol l^{-1} labeled amino acid at pH 10.

Inhibition in the absence of a K^+ gradient: BBMV in 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD and 100 mmol l^{-1} KSCN at pH 10, were preincubated with valinomycin (8 μg mg^{-1} protein) and resuspended to give a final reaction mixture containing 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD, 100 mmol l^{-1} KSCN, 10 mmol l^{-1} inhibiting amino acid and 0.5 mmol l^{-1} labeled amino acid at pH 10.

Uptake was stopped at 6 s and the values listed are mean uptake values at the end of this interval ± S.E.M. from triplicate BBMV preparations.

Asterisks denote values not significantly different from control uptake in the absence of inhibitory amino acid.

other countertransport experiments. Amino acids that were found to be the strongest and weakest inhibitors were used as elicitors in order to outline glutamate countertransport patterns (Table 2).

In order to estimate the binding affinity of glutamate, initial uptake rates as a function of $[\text{glutamate}]_{out}$ between 0.001 and 10.0 mmol l^{-1} were measured. These rates were very low and independent of $[\text{glutamate}]_{out}$ to within experimental error.

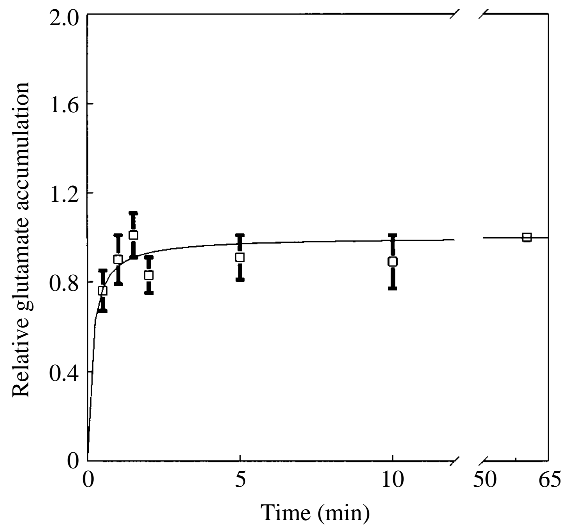

Fig. 7. Time course of labeled glutamate uptake elicited by 20 mmol l^{-1} glutamate. Conditions for countertransport are specified in the text. Intravesicular glutamate concentrations are expressed relative to the concentrations at equilibrium. The curve shown is a visual guide. When not given, the error bars were smaller than the symbols used.

Discussion

The initial rate of glutamate uptake was accelerated by a K^+ gradient (Fig. 2), and glutamate concentration in larval *Manduca sexta* midgut BBMV reached a maximum of 2–3 times the equilibrium value in the presence of an initial alkali ion gradient (Fig. 2). No accumulation was observed, either in the absence of an alkali cation or in the absence of an alkali ion electrochemical gradient (Fig. 2). We infer from these results that glutamate is symported since its flux is (a) coupled to alkali ion entry and (b) driven by the electrochemical gradient of the cation.

In contrast to its effect on the Na^+-dependent mechanism in crustacean hepatopancreatic BBMV (Balon and Ahearn, 1991), a Cl^- gradient does not seem to increase the glutamate uptake rate (Fig. 2). This may not be surprising, considering that the concentration of Cl^- in the anterior midgut fluid is about 25-fold lower than that of K^+ (Dow *et al.* 1984). In any event, the maximum accumulation reached with a given alkali cation is pH-dependent. Thus, glutamate uptake is stimulated by both K^+ and Na^+ gradients, with increasing specificity for K^+ at higher pH values. A Li^+ gradient was able to stimulate glutamate uptake at pH 8.9 but not at pH 9.7 nor at pH 10.5, whereas Rb^+ and Cs^+ gradients were ineffective at any of the pH values examined (Figs 3, 4). These results indicate that, at physiological pH values, glutamate symport occurs preferentially with K^+, which is present *in vivo* at far higher concentrations than is Na^+ (179±23 mmol kg^{-1} wet mass *versus* $<$1 mmol kg^{-1} wet mass; Dow *et al.* 1984).

Wolfersberger (1993) found no evidence for above-equilibrium accumulations of glutamate in larval *Lymantria dispar* BBMV with inwardly directed alkali ion gradients. BBMV from the larval midgut of *Pieris brassicae* (Wolfersberger *et al.* 1987) were

Table 2. *Relative countertransport accumulation of glutamate elicited by selective amino acids*

Elicitor	Relative accumulation
Lysine	0.88±0.08
Phenylalanine	0.87±0.04
Aspartate	0.97±0.18
D-Glutamate	0.76±0.03
Glutamate 5-methyl ester	0.77±0.09
Control	0.59±0.08

BBMV were preloaded with 20 mmol l^{-1} elicitor amino acid in 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD and 100 mmol l^{-1} KSCN at pH 10 and preincubated with valinomycin (8 μg mg^{-1} protein). The vesicles were resuspended in a reaction mixture containing the following final concentrations: 100 mmol l^{-1} mannitol, 50 mmol l^{-1} AMPD, 100 mmol l^{-1} KSCN and 0.5 mmol l^{-1} labeled amino acid at pH 10.

Intravesicular glutamate concentrations were determined at 90 s and 60 min.

Mean countertransport accumulations with their standard errors were determined by dividing uptake at 90 s by equilibrium uptake (at 60 min) for triplicate BBMV preparations.

similar to mammalian systems in that modest accumulations of glutamate were found in the presence of Na^+ but not K^+. Working at pH 7.4 with larval *P. cynthia* midgut BBMV, Giordana *et al.* (1989) demonstrated that glutamate uptake was higher in the presence of Na^+ than in the presence of either Li^+, Rb^+ or Cs^+ as the symported cation. Accumulations twice that at equilibrium were observed. In these respects, *M. sexta* seems to resemble *P. cynthia* rather than the other lepidopterans.

The relationship between potential difference and glutamate uptake

Fig. 5 reveals that the application of a transmembrane PD between −59 and +59 mV accelerates amino acid uptake in the order lysine>leucine⩾glutamate. Fluorescence spectroscopic experiments with a potential-sensitive dye had earlier shown that glutamate uptake rates were PD-independent between −50 and −110 mV (Parthasarathy and Harvey, 1994). In contrast, neutral amino acid uptake rates increased as the vesicle was depolarized. Recollecting that amino acid uptake across the BBM is driven by a PD *in vivo*, a feature that can be reproduced *in vitro* (Fig. 5), the insensitivity of glutamate uptake rates to PD strongly suggests that symport may not be an important means of glutamate transport across the BBM in the living organism. Glutamate symport has been found to be electroneutral in other tissues, such as *P. cynthia* midgut (Giordana *et al.* 1989) and mammalian intestine (Corcelli *et al.* 1982; Schneider *et al.* 1980). A number of factors, including an electroneutral limiting step or changes in substrate stoichiometry (compensating for changes in amino acid or carrier charge), could be responsible for the invariant glutamate uptake rate.

Uptake inhibition and countertransport of glutamate

Amino acid symport systems can often be distinguished from one another by their inhibition and countertransport patterns (Christensen, 1984). Inhibition of amino acid uptake can arise from (a) competitive inhibition, (b) non-competitive inhibition (symport

of the inhibitor through a different symporter dissipates the cation electrochemical gradient) or (c) uncompetitive inhibition. Cases a and b can be resolved by inhibition experiments in the absence of a salt gradient. Cases a and c can be resolved by countertransport experiments.

Inhibition in the presence of a K^+ gradient

Each of the 20 naturally occurring amino acids depressed glutamate uptakes below that of the control in the presence of a K^+ gradient (Table 1). Tryptophan, aspartate and phenylalanine inhibited glutamate uptake by 72 %, 67 % and 62 %, respectively. These results could reflect competition either for a glutamate-binding site or for the K^+ gradient. At the other end of the spectrum, lysine and 5-MG inhibited uptake only by 12 % and 6 %, respectively. Other amino acids, including D-glutamate and DMG, inhibited uptake by less than 50 %. These results suggest that lysine and 5-MG do not compete with glutamate for the K^+ gradient. There is little correspondence between the salt gradient data reported in Table 1 and those reported in rabbit jejunal BBMV at pH 6.0 by Maenz *et al.* (1992).

Inhibition in the absence of a K^+ gradient

The degree of inhibition of glutamate uptake was lower without, than with, a K^+ gradient. Inasmuch as the amino acids examined, including glutamate and aspartate, were found to inhibit glutamate uptake to similar extents, it appears that the putative glutamate-specific symporter does not have a well-defined set of inhibitors.

Countertransport

Countertransport experiments were attempted with several amino acids, chosen to represent either strong or weak inhibitors of glutamate uptake. The relative accumulations ranged from 0.77±0.09 for 5-MG to 0.97±0.18 for aspartate. At no time did any of the amino acids examined elicit intravesicular labeled glutamate uptake greater than equilibrium values. Glutamate itself elicited no accumulation of its labeled congener (Fig. 7). Hanozet *et al.* (1989) found that unlabeled glutamate elicited a relative accumulation of about 1.5 that of labeled glutamate in *P. cynthia* at neutral pH. We conclude that none of these amino acids (including glutamate) is symported by a putative glutamate-specific symporter.

In *M. sexta*, glutamate uptake resembles neutral amino acid symport in that it is K^+-specific above pH 9.7, but differs in that (a) glutamate uptake rates are pH-independent between pH values of 8 and 10, (b) the process is electroneutral, (c) unlabelled glutamate appears to be unable to elicit counterflow accumulations of its labeled counterpart and (e) a well-defined set of inhibitors is absent. The absence of a symporter specific for glutamate would serve to explain the low uptake rate of this amino acid as well as the non-specific inhibition, the high K_m values and the failure of intravesicular glutamate to elicit labeled glutamate uptake. The accumulations observed in the presence of an alkali ion gradient may reflect glutamate symport *via* symporters of other amino acids. The binding constant of glutamate in *P. cynthia* was 3–5 times higher than that of the neutral amino acids and the V_{max} was between 15 and 50 % of that of the neutral amino acids

(Giordana *et al.* 1989). However, at pH 7.4, the K_m and V_{max} of aspartate symport in *M. sexta* (Reuveni and Dunn, 1993) were similar to those of leucine. The differences between aspartate and glutamate symport at the same pH remain to be investigated. In any event, glutamate symport in lepidopterans, including *M. sexta,* seems to be marginal.

Glutamate is present at a very low concentration in the haemolymph but is relatively abundant in the midgut lumen and within the midgut epithelial cells of larval *P. cynthia* (Giordana *et al.* 1989) and *B. mori* (Parenti *et al.* 1985). Considering the pivotal position that glutamate occupies in intermediary metabolism, it is likely that glutamate is mainly synthesized by endogenous mechanisms so that a pathway optimized for glutamate uptake from the diet may not have evolved. Glutamate *synthesis*, rather than symport, may account for the high concentrations of glutamate in the epithelial cells.

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