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CHARACTERISTICS OF GLUTAMIC ACID TRANSPORT BY RABBIT INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

EFFECTS OF Na^+-, K^+- AND H^+-GRADIENTS

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In the presence of a Na^+-gradient (out > in), L-glutamic acid and L-and D-aspartic acids were equally well concentrated inside the vesicles, while no transport above simple diffusion levels was seen by replacement of Na^+ by K^+. Equilibrium uptake values were found inversely proportional to the medium osmolarity, thus demonstrating uptake into an osmotically sensitive intravesicular space. The extrapolation of these lines to infinite medium osmolarity (zero space) showed only a small binding component in acidic amino-acid transport. When the same experiment was performed at saturating substrate concentrations, linear relationships extrapolating through the origin but showing smaller slope values were recorded, thus indicating that the binding component could be more important than suspected above. However, binding to the membrane was neglected in our studies as it was absent from initial rate measurements. Na^+-dependent uphill transport of L-glutamic acid was stimulated by K^+ present on the intravesicular side only but maximal stimulation was recorded under conditions of an outward K^+-gradient (in > out). Quantitative and qualitative differences in the K^+ effect were noted between pH 6.0 and 8.0. Initial uptake rates showed pH dependency in Na^+-(out > in) + K^+-(in > out) gradient conditions only with a physiological pH optimum between 7.0 and 7.5. It was also found that a pH-gradient (acidic outside) could stimulate both the Na^+-gradient and the $Na^+ + K^+$-gradient-dependent transport of L-glutamic acid. However, pH- or K^+-gradient alone were ineffective in stimulating uptake above simple diffusion level. Finally, it was found that increased rates of efflux were always observed with an acidic pH outside, whatever the conditions inside the vesicles. From these results, we propose a channel-type mechanism of L-glutamic acid transport in which Na^+ and K^+ effects are modulated by the surrounding pH. The model proposes a carrier with high or low affinity for Na^+ in the protonated or unprotonated forms, respectively. We also propose that K^+ binding occurs only to the unprotonated carrier and allows its fast recycling as compared to the free form of the carrier. Such a model would be maximally active and effective in the intestine in the in vivo physiological situations.

Introduction

Studies on the characterization of dicarboxylic amino-acid transport by the small intestinal mucosa in vivo or in vitro have been impaired by the rapid transamination of these amino acids in intact preparations [1,2]. However, the existence of a specific, sodium-dependent transport system for acidic amino acids was clearly demonstrated by the work of Schultz et al. [3]. Also, the presence of a system shared by L-glutamic and L-aspartic acids

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

is apparent from the report of two forms of dicarboxylic aminoaciduria [4,5], one of them affecting both renal and intestinal carriers [4]. Unexpectedly, the introduction of brush-border membrane vesicles in the field of membrane transport, which allow the dissociation of transport from metabolic events [6], has mostly stimulated the reinvestigation of the characteristics of dicarboxylic amino-acid transport in the kidney [7–12]. To our knowledge, only one paper has dealt with this problem in the small intestine [13]. All these studies showed a strict requirement for Na^+ in the carrier-mediated process and proposed an electroneutral cotransport model according to the gradient hypothesis [14–16] with a stoichiometry of one Na^+ per glutamate anion transported [10,13]. It was also shown, in renal brush-border membrane vesicles, that a K^+-gradient ($[K^+]_i > [K^+]_o$) could drive the uphill transport of acidic amino acids in the presence of Na^+ ($[Na^+]_o = [Na^+]_i$) or increase the Na^+-gradient-dependent ($[Na^+]_o > [Na^+]_i$) uptake of L-glutamate [8,9,12]. A Na^+-glutamate symport/K^+ antiport has been hypothesized with stoichiometries in relation with an electroneutral [9,12] or an electrogenic [8] system. Since the rheogenicity has been unequivocally demonstrated in the rat by electrophysiological techniques [17], it could be species-specific [12]. A role for H^+ (OH^-) has also been postulated in rabbit kidney [12]. Such studies are lacking for the intestine.

In this paper, we examine the characteristics of glutamic-acid transport by rabbit intestinal brush-border membrane vesicles with special emphasis on the possible effects of Na^+, K^+ and H^+ (OH^-) on transport. Preliminary account of this work has been published in abstract form [18].

Materials and Methods

Chemicals. All salts and chemicals for buffer preparation were of the highest purity available. Unlabeled amino acids were obtained from Sigma Chemical Company (L-glutamic acid), BDH Chemicals (L-aspartic acid) and Aldrich Chemical Company (D-glutamic and D-aspartic acids). All labeled compounds were from New England Nuclear Corporation as follows: L-[U-^{14}C]glutamic acid (271–294 mCi/mmol), L-[U-^{14}C]aspartic acid (219 mCi/mmol), D-[2,3-^{3}H]aspartic acid (20 Ci/mmol), D-[U-^{14}C]glucose (348–360 mCi/mmol), D-[1-^{14}C]mannitol (45 mCi/mmol) and D-[1-(n)-^{3}H]mannitol (17–27.4 Ci/mmol).

Preparation of brush-border membrane vesicles. The small intestine was removed from 1.8–2.3 kg male New Zealand white rabbits (Ferme Cunicole Léonard, Ste Cholastique, Québec) and flushed with ice-cold 0.9% NaCl. The mucosa from the jejunum was scraped with a spatula on a cold glass plate. Brush-border membranes were purified by the calcium chloride precipitation method of Schmitz et al. [19] and brush-border membrane vesicles were obtained by the method of Hopfer et al. [6] as described previously for the mouse intestine [20] but with the following modifications. P_2 fractions were resuspended in the final resuspension buffer for vesicles and vesicles were obtained as a P_4 fraction after two 15 min centrifugations at $2000 \times g$ and $31\,000 \times g$, respectively. Based on sucrase and alkaline phosphatase activities and on glucose uptake determinations, these modifications allow a faster recuperation of vesicles without any loss in efficiency. Enrichment factors of the above enzyme activities with respect to the homogenate were 13.4 ± 1.7 and 8.5 ± 0.6, respectively (mean of 12 determinations).

Transport studies. Uptake studies were carried out by the rapid-filtration technique of Hopfer et al. [6] as previously described for the mouse intestine [20]. The freshly purified brush-border membrane vesicles were resuspended to a final protein concentration of 8–12 mg/ml with the final resuspension buffer and an aliquot (400–600 μg protein) was added to the incubation medium kept at room temperature (20 °C) to start the transport experiments. The compositions of the final resuspension buffers for vesicles and the final concentrations in incubation media will be indicated in the legends of the figures. At time intervals, aliquots were taken from the incubation mixture and poured in 1 ml quenched ice-cold stop-solution (composition adjusted to experimental conditions as to match the final concentrations of the different species in the incubation media). The quenched mixture was then filtered on a pre-wetted and chilled 0.45 μm nitrocellulose filter (Sartorius SM 11306) and washed with 4 ml of nonradioactive ice-cold stop solution. Filters were dissolved and ^{3}H and ^{14}C radioactivities were determined as

previously described [20] using a LKB (Model 1215 rackbeta II) scintillation counter. Results are expressed as nmol solute uptake/mg protein. Initial rates of uptake have been computed by linear-regression analysis or polynomial fitting of uptake time-courses from zero to 1 min (four points) using an Apple II desk computer and a curve fitter program (P.K. Warme, Copyright (C) 1980, Interactive Microwave Inc.).

Assays. Marker enzymes for the brush-border membrane, sucrase (EC 3.2.1.48) and alkaline phosphatase (EC 3.1.3.1), were routinely essayed by the method of Dahlqvist [21] as modified by Llyod and Whelan [22] and by the method of Eichholz [23], respectively. Protein was measured according to Lowry et al. [24] with bovine serum albumin as standard.

Results

Uptake time courses of dicarboxylic amino acids

Fig. 1 shows the zero-trans entry of 50 μM L-[^{14}C]glutamic and or L-[^{14}C]aspartic acid or D-[^{3}H]aspartic acid as a function of time when measured in initial gradient conditions of NaCl (top curves) or KCl (bottom curves). All substrates were taken up to the same extent by the vesicles and it is apparent, from the observed overshoots in the presence of a NaCl gradient as compared to a KCl gradient, that they were concentrated inside the vesicles against a chemical gradient by a Na^+-dependent mechanism. The uptake was linear up to 1 min, reached a maximum around 10 min and then decreased so slowly towards equilibrium values that they were not reached at 90 min. In the other experiments, equilibrium values were recorded at 3 h. It has to be noted, however, that different plateau values were always recorded between NaCl and KCl conditions (up to 12 h after initiation of uptake), so that some binding to the membrane was suspected in the presence of Na^+. Other experiments have been conducted in which the 100 mM KCl has been replaced by 100 mM choline chloride, 200 mM mannitol, or 100 mM sodium glutamate. It then appeared that the curves obtained under such conditions were all indistinguishable (results not shown), so that the curves drawn in K^+-gradient conditions in Fig. 1 are likely to represent simple diffusion.

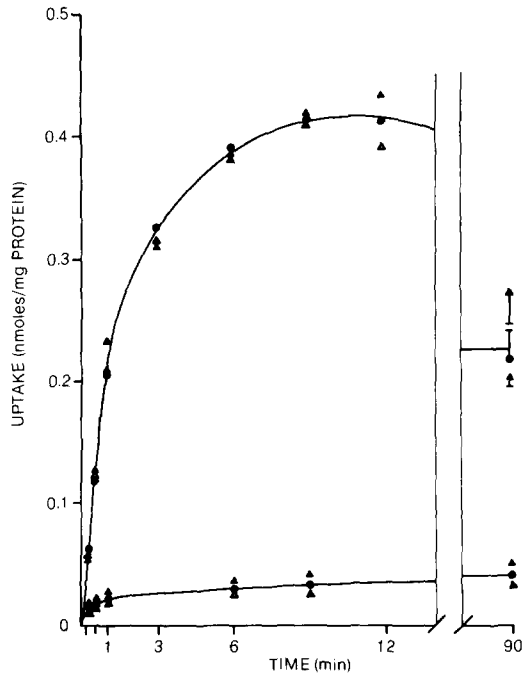

Fig. 1. Uptake time courses of 50 μM L-glutamic acid (●), L-aspartic acid (▲) and D-aspartic acid (△) in 100 mM NaCl (top curves) or KCl (bottom curves). Final resuspension buffer for vesicles: 10 mM Tris-Hepes buffer (pH 7.5), 300 mM mannitol and 0.1 mM $MgSO_4$. Final concentrations in the incubation medium (250 μl): 10 mM Tris-Hepes buffer (pH 7.5), 100 mM mannitol, 0.1 mM $MgSO_4$, 100 mM NaCl or 100 mM KCl, 50 μM substrates with 3.63, 2.74 or 5.48 μCi of L-[U-^{14}C]glutamic acid, L-[U-^{14}C]aspartic acid or D-[2,3-^{3}H]aspartic acid, respectively. Each point is the mean of three experiments with different preparations of vesicles.

Uptake as a function of osmolarity

In order to evaluate binding contribution to dicarboxylic amino-acid transport, equilibrium uptake was measured at increasing osmolarities in the incubation medium by varying the extravesicular concentration of mannitol. As shown in Fig. 2 for 50 μM substrate concentrations (upper curves), this uptake was inversely proportional to the medium osmolarity and only minimal binding could be evaluated by extrapolation to infinite medium osmolarity (internal volume = 0). In order to remove any specific binding to the membrane from equilibrium uptake values, the same experiment was done at saturating substrate concentrations of 100 mM (lower curves in Fig. 2). In these conditions, extrapolation to infinite osmolarities

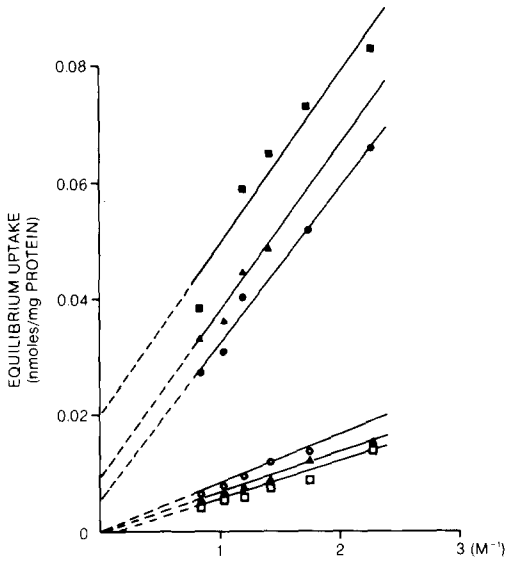

Fig. 2. Effect of medium osmolarity on equilibrium uptake of L-glutamic acid (●,○), L-aspartic acid (▼,▽) and D-aspartic acid (■,□) in the presence of 100 mM NaCl (●,▼,■) or 100 mM sodium D-glutamate (○), L-aspartate (▽) and D-aspartate (□). Final resuspension buffer for vesicles: 10 mM Tris-Hepes buffer (pH 7.5), 50 mM mannitol and 0.1 mM $MgSO_4$. Final concentrations in the incubation media (150 μl): 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 50 μM substrates with 2.18, 1.65 or 3.27 μCi of L-[U-^{14}C]glutamic acid, L-[U-^{14}C]aspartic acid or D-[2,3-^{3}H]aspartic acid, respectively, 100 mM NaCl (closed symbols) or 100 mM NaOH-neutralized substrates (open symbols) and enough mannitol to get the desired osmolarities. Each point is the mean of two 50 μl aliquots taken up from the mixture after 4 h incubation. Linear-regression analysis was performed on an Apple II desk-computer using a curve-fitter program (P.K. Warme, Copyright © 1980, Interactive Microware Inc.). Correlation coefficients (r) and slopes were, respectively: 0.982 and 0.0294 (■), 0.988 and 0.0281 (▲), 0.997 and 0.0268 (●), 0.996 and 0.00838 (○), 0.997 and 0.00696 (△), and 0.981 and 0.00687 (□).

were not different from zero. However, different slopes were obtained as compared to the previous experimental conditions even though the same internal osmolarity (0.27 M) was used for both. We must conclude that different apparent internal intravesicular volumes have been recorded, thus demonstrating binding in nonsaturating conditions. By dividing the slopes by the internal osmolarity, it is possible to determine intravesicular spaces corresponding to 0.104 ± 0.0048 and 0.0274 ± 0.0031 nmol/mg protein at 50 μM and 100 mM substrate concentrations, respectively, and then to calculate a binding contribution of 0.0766 ± 0.0078 nmol/mg protein by substraction. In the following experiments, uptake values were not corrected for this binding as it represents only a small fraction of uptake. Moreover, it should be noted that this binding is likely to represent internal binding as extrapolation of uptake curves from the early time points goes through the origin. Thus, binding is a late event that should not influence significantly the interpretation of the results presented in the following sections.

Interactions between Na^+ and K^+ for glutamic acid uptake at different pH values

The effects of Na^+ and K^+ on glutamic acid transport have been investigated as described in the legend of Fig. 3, and the following results have been obtained. No transport was observed in the complete absence of Na^+ (choline in = choline out = 208 mM) or in the presence of K^+ alone (in: choline 208 mM/out: choline = 108 mM, KCl = 100 mM or in: KCl = choline = 104 mM/out: choline = 204 mM, KCl = 4 mM or choline = 104 mM, KCl = 104 mM). Also, an outwardly directed Na^+-gradient was unable to increase uptake above the simple diffusion level either in the absence or presence of K^+ (in: NaCl = choline = 104 mM/out: choline = 204 mM, NaCl = 4 mM or choline = 104 mM, KCl = 100 mM, NaCl = 4 mM or in: NaCl = KCl = 104 mM/out: choline = 200 mM, KCl = NaCl = 4 mM or choline = 100 mM, KCl = 104 mM, NaCl = 4 mM). These eight situations have been meaned and called simple diffusion on Fig. 3. Glutamic acid uptake was, however, elicited with Na^+ equilibrated on both sides of the membrane (in: NaCl = choline = 104 mM/out: NaCl = choline = 104 mM), and K^+ outside (in: NaCl = choline = 104 mM/out: NaCl = 104 mM, KCl = 100 mM, choline = 4 mM) or equilibrated (in: NaCl = KCl = 104 mM/out: NaCl = KCl = 104 mM) did not modify it. These three situations appear as Na^+ equilibrated in Fig. 3. However, when an outwardly directed K^+-gradient was present (in: NaCl = KCl = 103 mM/out: NaCl = 104 mM, KCl = 4 mM, choline = 100 mM), increased uptake rates were observed. The effect was small at pH 6.0 but a slight overshoot was present at pH 8.0. An overshoot was also induced by the presence of an inwardly di-

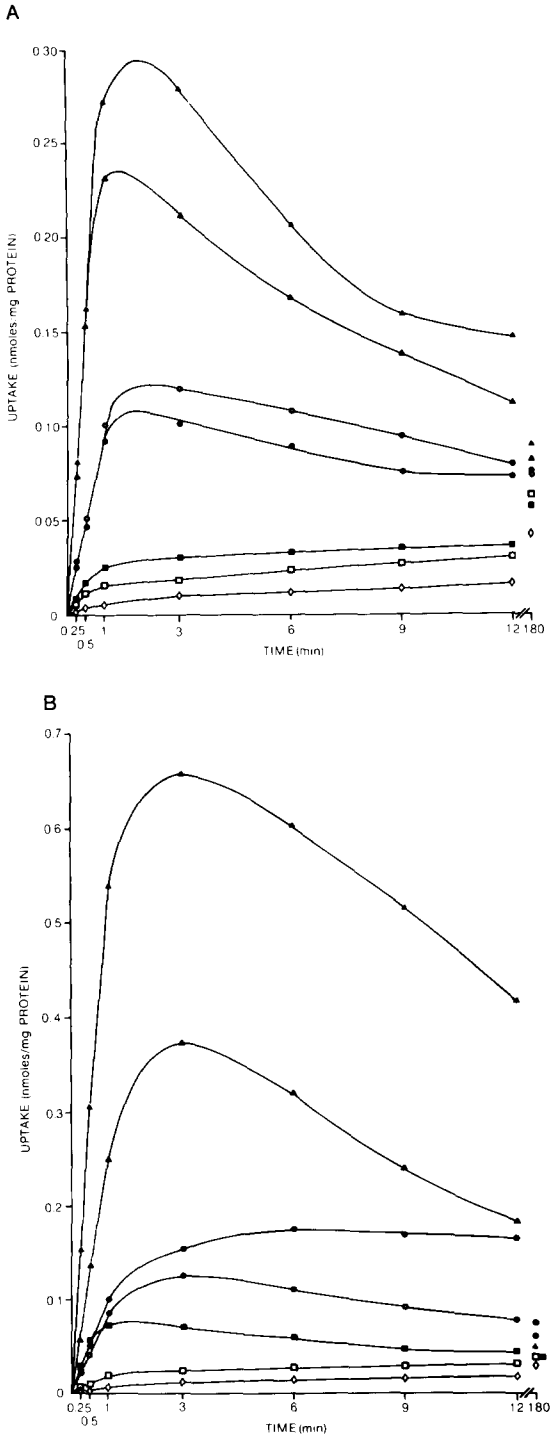

Fig. 3. Interactions between Na^+ and K^+ for glutamic acid uptake at pH 6.0 (A) and 8.0 (B). Final resuspension buffer for vesicles: 50 mM Tris-Mes (pH 6.0) or Tris-Hepes (pH 8.0), 0.1 mM $MgSO_4$, and either 208 mM choline chloride, or 104 mM KCl and 104 mM choline chloride, or 104 mM NaCl and 104 mM choline chloride, or 104 mM NaCl and 104 mM KCl. 10 μl of these vesicles were introduced at time zero in 240 μl of the different resuspension buffers containing 3.63 μCi of L-[U-^{14}C]glutamic acid (50 μM final). In these conditions, 16 ionic combinations were generated at each pH and analyzed for glutamic acid uptake. Only the significant results have been plotted. This is a representative experiment (out of two) performed the same day on the same preparation of vesicles for comparison purposes. Symbols have the following meanings (see text for exact composition inside and outside the vesicles in each case): simple diffusion (◇), Na^+-equilibrated (□), Na^+-equilibrated with outward K^+-gradient (■), inward Na^+-gradient (●), inward Na^+- and K^+-gradients (○), inward Na^+-gradient with K^+-equilibrated (△), inward Na^+-gradient with outward K^+-gradient (▲).

rected Na^+-gradient (in: choline = 208 mM/out: NaCl = 100 mM, choline = 108 mM) as already described (Fig. 1) and the effect was slightly increased at pH 6.0 or decreased at pH 8.0 by superposition of a K^+-gradient in the same direction (in: choline = 208 mM/out: NaCl = 100 mM, KCl = 100 mM, choline = 8 mM). However, the Na^+-dependent uphill transport of glutamic acid was greatly stimulated with K^+ equilibrated on both sides of the membrane (in: KCl = choline = 104 mM/out: NaCl = 100 mM, KCl = 104 mM, choline = 4 mM) and still further increased when an outwardly K^+-gradient was present (in: KCl = choline = 104 mM/out: NaCl = 100 mM, KCl = 4 mM, choline = 104 mM). At pH 8.0, the last effect was visible on both the initial rate and the amplitude of the overshoot but only maximum overshoot values were affected at pH 6.0. It also has to be noted that stimulations by K^+ were more important at the basic pH (note the scale difference between Fig. 3A and B).

Effect of pH on L-glutamic acid uptake

The pH effect noted in the above studies was documented by analyzing the uptake of L-glutamic acid at different pH values and the results of such an experiment are shown in Figs. 4 and 5. In Na^+-gradient conditions (Fig. 4A), a pH variation from 6.0 to 8.0 did not influence greatly the initial rates of uptake (Fig. 5). However, a pH of 9 was clearly inhibitory (Figs. 4A and 5). The main effect of increasing the pH was to delay the time at which the overshoot was maximum and to increase the amplitude of the overshoot. Decreased rates of

efflux were also apparent (Fig. 4A). The picture was quite different in Na^+-(out > in) and K^+-(in > out) gradient conditions (Fig. 4B). A clear effect on initial rates of uptake was obtained in these conditions and a pH optimum of 7.0–7.5 can be determined (Fig. 5). Also, the time delay in maximum overshoot values was not apparent before pH 7.5 and the differences between the amplitudes of the overshoots were not so important between pH 6.5–8.0. Decreased rates of efflux at basic pH values were also more apparent. For comparison, Fig. 4C shows the effect of pH on glucose transport in Na^+-gradient conditions. Increasing the pH from 6.0 to 9.0 did not affect either the initial rates of uptake or the efflux rates. However, a time delay in maximum uptake values and an increased amplitude of the overshoots were observed.

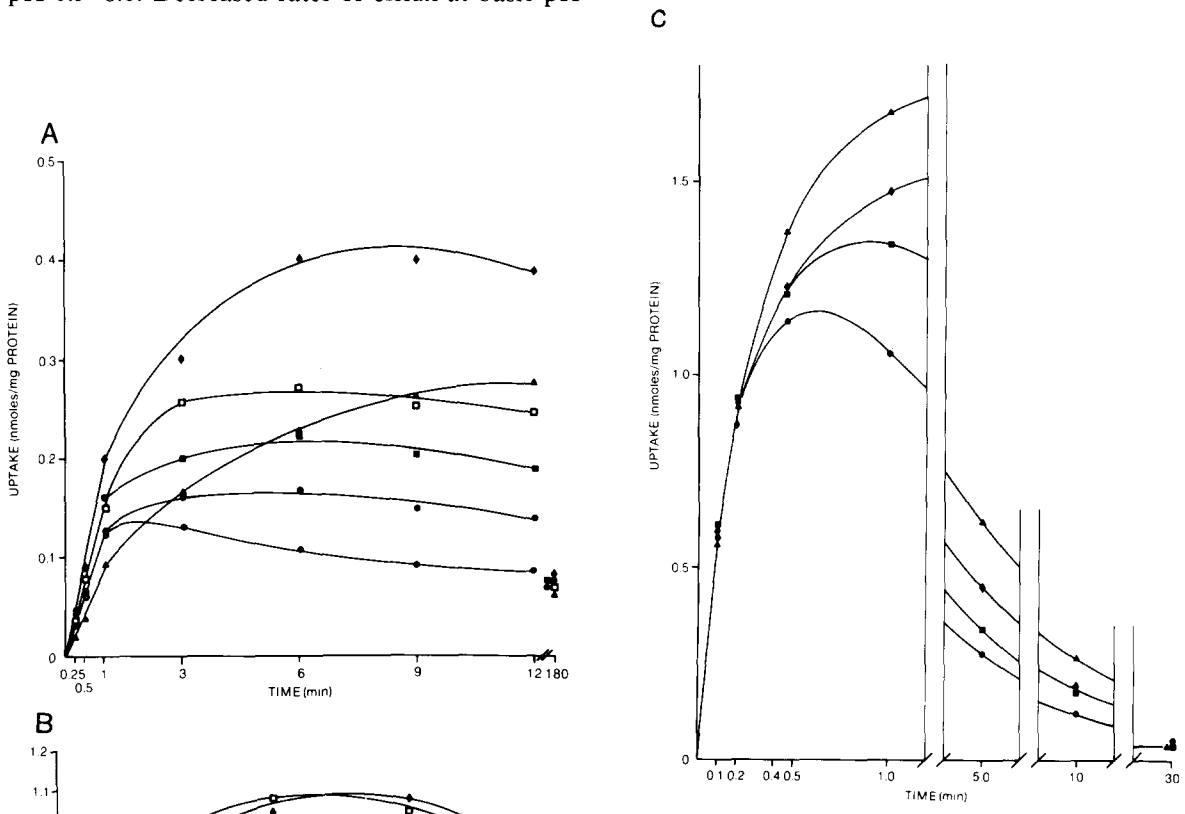

Fig. 4. Effects of pH on glutamic acid (A, B) and glucose (C) uptake in Na^+-gradient (A, C) and Na^+- $+K^+$-gradient (B) conditions. Final resuspension buffer for vesicles: 10 mM Mes-Tris (pH 6.0) (●) or 6.5 (○) or 10 mM Hepes-Tris (pH 7.0) (■) or 7.5 (□) or 10 mM Tris-Hepes (pH 8.0) (◆) or 9.0 (△), 0.1 mM $MgSO_4$, 208 mM choline chloride (A, C) or 104 mM KCl and 104 mM choline chloride (B). Final concentrations in the incubation media (250 μl): 10 mM Mes-Tris (pH 6.0) (●) or 6.5 (○) or 10 mM Hepes-Tris (pH 7.0) (■) or 7.5 (□) or 10 mM Tris-Hepes (pH 8.0) (◆) or 9.0 (△), 0.1 mM $MgSO_4$, 100 mM NaCl and 108 mM choline chloride (A, C) or 100 mM NaCl, 4 mM KCl and 104 mM choline chloride (B) and 50 μM L-glutamic acid with 3.63 μCi of L-[U-^{14}C]glutamic acid or 50 μM D-glucose with 3.33 μCi of D-[U-^{14}C]glucose. Points shown are individual data points from the same preparation of vesicles and are representative of the four (A) and the two (B, C) experiments performed in the same conditions.

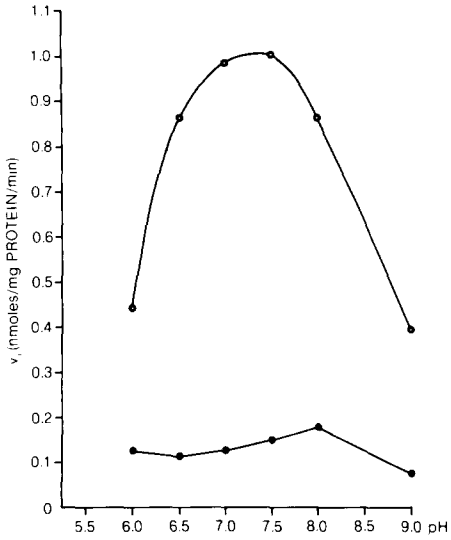

Fig. 5. Effect of pH on initial rates of L-glutamic acid uptake in Na^+-gradient (●) and $Na^+ + K^+$-gradient (○) conditions. Initial rates have been computed from Fig. 4 by linear-regression or polynomial fitting of the points up to 1 min (including 0), as discussed under Material and Methods.

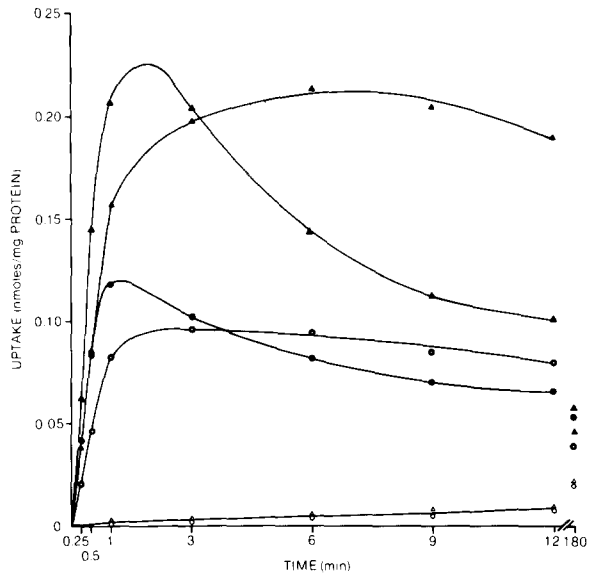

Fig. 6. Effect of pH gradients on glutamic acid uptake in Na^+-gradient (out > in) conditions. Final resuspension buffers for vesicles: 50 mM Tris-Mes (pH 6.0) (●,❍,○), or Tris-Hepes (pH 8.0) (▲,▲,△), 0.1 mM $MgSO_4$ and 208 mM choline chloride. Final concentrations in the incubation media (250 μl): 50 mM Tris-Mes (pH 6.0) (●,▲,△) or Tris-Hepes (pH 8.0) (○,▲,△), 0.1 mM $MgSO_4$, 108 mM choline chloride, 100 mM NaCl (●,❍,▲,▲) or choline chloride (○,△) and 50 μM glutamic acid with 3.63 μCi of L-[U-^{14}C]glutamic acid. Points shown are individual data points and are representative of the two experiments performed in the same conditions.

Effects of pH gradients on glutamic acid uptake

We have investigated the effect of pH-gradients on glutamic-acid uptake in different ionic conditions as described in the legends of Figs. 6 and 7. These experiments have been performed on the same day with the same preparation of vesicles for comparison purposes. In Fig. 6 are presented the results in Na^+gradient conditions and the following observations have been made. First, as already noted in Fig. 4A, initial rates of glutamic acid uptake were not affected by the pH in the incubation medium up to 0.5 min but higher overshoot values were recorded at pH 8.0 as compared to pH 6.0. The effect seems partly due to higher efflux rates observed at the more acidic pH. Second, inwardly directed H^+-gradient-stimulated initial rates of transport and gave the highest accumulation ratio observed in these experiments. On the contrary, outwardly directed H^+-gradient decreased the initial rate of transport and gave the lowest overshoot values. Again, higher efflux rates were obtained with an acidic pH outside. Finally, pH-gradient alone, whatever its direction, was ineffective in driving glutamic-acid uptake above simple diffusion values (Fig. 6, lower curve). In Na^+-gradient conditions and either inwardly directed K^+-gradient or K^+ equilibrated on both sides of the membrane, very similar results were obtained (results not shown) with highest uptake rates and highest overshoot values recorded for an inwardly directed H^+-gradient, while the reverse was true for an outwardly directed H^+-gradient. The only observed difference was a slight decrease in uptake rates at more basic pH even if higher overshoot values were seen at this pH. Again, this effect seems partly due to higher efflux rates obtained with more acidic pH outside. As in Fig. 6, pH-gradients alone had no effect on uptake. Fig. 7 shows the results obtained when pH-gradients were combined with Na^+- and K^+-gradients of opposite directions (Na^+ outside, K^+ inside). A somewhat different picture emerged in these conditions. First, initial rates of glutamic acid transport were stimulated by the basic pH as compared to the

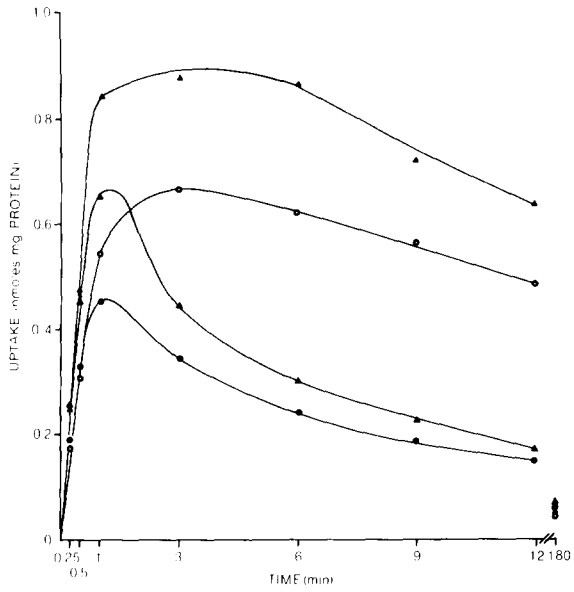

Fig. 7. Effect of pH-gradients on glutamic acid uptake in Na^+-(out > in) and K^+-(in > out) gradient conditions. Final resuspension buffers for vesicles: 50 mM Tris-Mes (pH 6.0) (●,○) or Tris-Hepes (pH 8.0) (▲,△), 0.1 mM $MgSO_4$, 104 mM choline chloride and 104 mM KCl. Final concentrations in the incubation media (250 μl): 50 mM Tris-Mes (pH 6.0) (●,△) or Tris-Hepes (pH 8.0) (▲,○), 0.1 mM $MgSO_4$, 108 mM choline chloride, 100 mM NaCl and 50 μM glutamic acid with 3.63 μCi of L-[U-^{14}C]glutamic acid. Points shown are individual data points from the same preparation of vesicles as used in Fig. 6 and are representative of the two experiments performed in the same conditions.

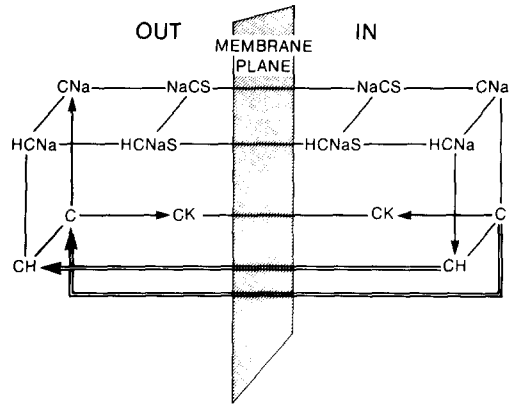

Fig. 8. A possible mechanism for glutamic acid transport in rabbit jejenum. The carrier can exist under protonated (CH) or unprotonated (C) forms. Only the protonated form can bind Na^+ with high affinity, while only the unprotonated form can bind K^+ with high affinity. The full lines represent the fast equilibrium steps, while the single arrows represent more limiting steps, i.e., binding of Na^+ to the carrier at basic pH outside, debinding of Na^+ from HCNa at acidic pH inside and CK formation at acidic pH inside or outside. Double arrows are for the overall rate-limiting steps in a complete cycle (return of the protonated and unprotonated forms of the carrier). The affinity for glutamic acid is only controlled by Na^+ binding.

acidic one, an effect already noted in Fig. 4B. Second, an inwardly directed H^+-gradient was unable to stimulate the initial rate above values recorded at pH 8.0 and an outwardly directed pH-gradient was not inhibitory when compared to pH 6 values. However, higher efflux rates were observed when an acidic pH was present on the external side of the membrane. Finally, pH-gradients alone were unable to increase uptake above simple diffusion values (not shown as undistinguishable from the x axis).

Discussion

Recent studies have indicated that glutamine, rather than glucose, is the major respiratory substrate for the small intestine [25,26], so that L-glutamic acid is likely to play a central role in intestinal metabolism. Consequently, the uptake of this amino acid by the enterocyte is of crucial importance. Earlier in vivo and in vitro studies, using intact intestinal preparations, have failed to show net transport of anionic amino acids against a concentration gradient or tissue accumulation [1,2], and it is now clear that the rapid metabolism of these amino acids has precluded such demonstrations [25,26]. In order to circumvent this problem, Schultz et al. [3] have used initial rate data, and so, could demonstrate active transport of acidic amino acids. In this paper, we have reinvestigated the characteristics of dicarboxylic amino-acid transport using isolated jejunal brush-border membranes vesicles in which it is possible to eliminate completely interferences due to metabolism [6]. Such an approach has been widely used to study L-glutamic acid transport in the kidney [7–12] but only poorly in the small intestine [13].

The results presented in this paper demonstrate the presence of a Na^+-dependent, carrier-mediated transport process for acidic amino acids in rabbit jejunal brush-border membranes. First, the presence of an inwardly directed Na^+-gradient

could elicit a 10-fold accumulation of substrates over medium concentrations inside the vesicles (Fig. 1). Also, when Na^+ was present at the same concentration inside and outside the vesicles (Fig. 3), a small stimulation of uptake was observed but no overshoot was recorded. Finally, when NaCl was replaced by equivalent concentrations of KCl, choline chloride, mannitol or sodium glutamate, uptake was very low and identical in all situations, so that it must represent simple diffusion as 100 mM sodium glutamate saturated the carrier-mediated process (Fig. 1). The conclusion is then that a Na^+-gradient provides the driving force for the uphill transport of acidic amino acids and that Na^+ is mandatory for uptake. This is in accordance with studies using rabbit [10,12] and rat [7] renal vesicles but at variance with earlier studies of Schultz et al. [3] with intact preparations of rabbit ileum or jejunum where unidirectional influxes were observed in the absence of Na^+. In the latter case, it is possible that the results obtained in Na^+-free conditions have been obscured by the presence of Na^+ trapped in the unstirred layer [27,28], a situation that would be amplified by the intracellular concentration of K^+ (see below).

The results of Fig. 1 also showed that the D- and L-stereoisomers of aspartic acid were transported as efficiently as L-glutamic acid with the same transport characteristics for Na^+-gradient dependency. This contrasts with the earlier findings that L-stereoisomers of aspartic acid were much more rapidly absorbed in vivo than are the D-stereoisomers [29], though preferential use of L-stereoisomers for metabolism can explain these earlier conclusions. Our results suggest that the three acidic amino acids share the same carrier, in accordance with results obtained in the rabbit [10,12] or rat [30] kidney and rat intestine [13]. However, the possibility that D- and L-stereoisomers are transported via different systems has been raised [17] and more data will prove necessary to get a definite answer to this question in our system.

When analyzing the results of Fig. 1, we also made the observation that equilibrium uptake values were always higher in Na^+ than in K^+ conditions, so that some binding was suspected. However, this binding component of uptake seems to be a late event as extrapolation of the early time points in uptake curves (up to 1 min) goes through the origin (this is true for all the figures presented in this paper). This impression was confirmed in Fig. 2 where extrapolation to infinite medium osmolarities gave low binding values of 5 pmol/mg protein for glutamic acid. This value contrasts with the 50 pmol/mg protein found for the same amino acid in rat intestinal brush-border membrane vesicles [13]. The difference may be due to different surface properties of rat and rabbit membranes [31]. Fig. 2 also demonstrates that the binding component found in our system is likely to be on the internal side of the vesicles or tightly bound to the membrane, as saturating the system with cold glutamic acid gave different slopes and thus different apparent intravesicular volumes. In the first case, the binding could be due to internal binding proteins (like cytoskeleton structures), while in the second, it could occur on membrane-binding proteins, the carrier itself being a likely candidate. From the Na^+-dependency of binding noted above, we favor the hypothesis of binding to the carrier. Such a Na^+-dependent binding of glutamate to a glutamate carrier has been reported in the mechanism of glutamate transport in *Escherichia coli* B [32,33]. Clearly, more studies are needed to clearify this point which is of crucial importance for mechanistic (limiting step) consideration (see below).

The specific effect of the Na^+-gradient in energizing the uphill transport of L-glutamic acid suggests that the translocation of this amino acid was coupled to the flux of Na^+, as observed in other systems [7,10,13]. However, the question as to whether it represents the only driving force in energizing the intravesicular accumulation of L-glutamic acid is of importance in the light of recent findings that outward K^+-gradient [8,9] or inward H^+-gradient (outward OH^--gradient) [12] could stimulate further the active transport of this amino acid. The effect of K^+ has been first analyzed at pH 6.0 and 8.0 where glutamic acid is dissociated to 98% as $[glutamate]^-$, so that the observed effects can only be attributed to events occurring at the membrane level (Fig. 3). It also has to be noted that Cl^- concentrations have been maintained equal on both sides of the membrane in these experiments in order to minimize membrane potential effects. In these conditions, we

were able to show that Na^+-gradient-driven L-glutamic acid uptake was only stimulated by intravesicular K^+ and that K^+-stimulation was greater under conditions of an outward K^+-gradient. The second effect was more important at pH 8.0 as compared to pH 6.0. This demonstration refutes the assertion that acidic amino-acid transport is not stimulated by a K^+-gradient in intestinal brush-border membrane vesicles (see discussion and footnote 2 in Ref. 9). Our results agree with a possible mechanism where K^+ efflux is coupled to the Na^+-dependent L-glutamic acid uptake [8,9,12] since K^+ stimulation was higher in the presence of a K^+-gradient ($[K_i^+] > [K_o^+]$) and was observed with an outward K^+-gradient in the absence of an inward Na^+-gradient ($[Na^+]_o = [Na^+]_i$). However, these two effects are pH-dependent and more visible at basic pH. Interesting enough are the observations made in Figs. 4 and 5 that initial rates of uptake were quite insensitive to pH variations in Na^+-gradient conditions but were highly dependent on pH when an outward K^+-gradient was superimposed on the inward Na^+-gradient. These pH dependencies are identical to those found by others [9,10] and suggest that K^+ effect(s) is (are) modulated by pH. It should be mentioned that inhibition of the Na^+-H^+ exchanger at basic pH values cannot explain the observed pH effects, as shown in Fig. 4C, where initial rates of glucose uptake were identical from pH 6.0 to 9.0. As calculated from Gunther and Wright [34], only 9 and 20% of total Na^+ flux would occur by Na^+/H^+ exchange at 100 mM NaCl concentration in the absence or in the presence of a pH-gradient, respectively. It also appears that efflux rates of glucose were not modified in these conditions. However, some effect was seen on the amplitude of the overshoots and one should be cautious when looking to maximal overshoot values (Figs. 4, 6 and 7).

These results can be integrated in a transport mechanism corresponding to the scheme of Fig. 8. In this model, the surrounding pH determines the existence of protonated (CH) and unprotonated (C) forms of the carrier which are interconvertible in a rapid equilibrium reaction (like it should be from the dissociation of an ionizable group on the protein). The unprotonated carrier has a low affinity for Na^+ and a high affinity for K^+ while the protonated carrier has a high affinity for Na^+ and a low (or no) affinity for K^+. Also, Na^+ binding allows substrate (S) binding with high affinity while K^+ binding does not, though permitting a fast recycling of the carrier as compared to its free forms, C and CH. In these conditions, the outside formation of CNa and the inside debinding of Na^+ from HCNa may become rate-limiting (as compared to the other reactions) in more basic or more acidic conditions, respectively. Intermediary complexes CS, KCS and HCS have been excluded from the model as they are not transported (Figs. 1 and 6). Finally, we have considered translocation of intermediary complexes CNa and HCNa as impossible from the results presented in this study. Also implicit in the model, from the rapid equilibrium through the membrane of complexes NaCS, HCNaS and CK, is the assumption that we are not dealing with a 'mobile-type' of carrier but with a 'channel-type' where conformation changes allow the translocation [35].

This model is compatible with the following results: (1) K^+ stimulation with ($[K_i^+] > [K_o]$) or without ($[K_i^+] = [K_o^+]$) K^+-gradient (Fig. 3); (2) higher stimulation by K^+ at pH 8.0 than 6.0 in K^+-gradient conditions (Fig. 3); (3) greater inhibitory effect of outside K^+ at pH 8.0 than 6.0 (Fig. 3); (4) outward K^+-gradient-induced overshoot in the absence of Na^+-gradient $[Na_i^+] = [Na_o^+]$ at pH 8.0 but only small effect at pH 6.0 (Fig. 3); (5) Independance of pH in Na^+-gradient conditions alone (Figs. 4 and 5); (6) higher activities of uptake obtained by increasing the pH in Na^+- (inward) and K^+- (outward) gradient conditions (Figs. 4 and 5); (7) increased rates of efflux with acidic conditions outside whatever inside conditions. The reverse is true with basic conditions outside (Figs. 4, 6 and 7). This is made possible by the dissociation of NaCS on the external side in acidic conditions, thus allowing accelerated exchange of internal S.

The model also predicts an increased rate of uptake in pH-gradient conditions (acidic outside) by shunting the two possible rate-limiting steps of Na^+-binding outside (at more basic pH) and Na^+-debinding inside (at more acidic pH). This was indeed observed (Fig. 6), except for outward K^+-gradient conditions (Fig. 7) where the increased rate of efflux observed with acidic pH

outside may have hidden the activatory effect. The model is also consistent with the inhibitory effect observed when these conditions were reversed (basic pH outside, acidic pH inside) as in Fig. 6. Again, the effect may have been less visible in Fig. 7 where efflux rates were decreased in conditions of basic pH outside. In these experiments, pH gradients had no influence in the absence of Na^+ (Fig. 6) or in the presence of K^+ alone (Fig. 7). This is at variance with reported effects by Sacktor [12] that a OH-gradient (in > out) effected the transient uphill uptake of L-glutamate, even when K^+ and Na^+ were deleted from the incubation media. The presence of an OH^--L-glutamate exchange system in intestinal brush borders is then ruled out. The model presented in Fig. 8 would predict an activatory effect of a pH-gradient (acidic outside) in the absence of Na^+ and K^+ driving forces, by shunting of the rate-limiting steps, as observed with kidney brush-border membrane visicles [12]. An inhibitory effect should be expected when the pH-gradient is reversed.

In conclusion, we have demonstrated active transport of glutamic acid in rabbit intestinal brush membranes by cotransport with Na^+ which is activated by internal K^+ and external H^+. A model has been proposed that accounts for the results presented and allows the design of new experiments for confrontation. On a physiological point of view, this system appears to be pH-regulated in vivo. In the jejunum, where an acidic microclimate is present at the external side of the membrane [36,37], the system will be maximally activated and the leak occurring in these conditions will be minimized by the rapid metabolism of glutamate [25]. In the distal ileum, with a more basic surface pH [37], the system is still very active but backflux is minimum in these conditions, thus keeping high intracellular concentrations of glutamic acid.

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