Biochimica et Biophysica Acta 861 (1986) 447–456
Elsevier

BBA 73307

Membrane potential dependency of glutamic acid transport in rabbit jejunal brush-border membrane vesicles: K^+ and H^+ effects

Alfred Berteloot

Membrane Transport Research Group, Department of Physiology, Faculty of Medicine, University of Montreal, C.P. 6208, succursale "A", Montreal, Quebec H3C 3T8 (Canada)

(Received 28 May 1986)

Key words: Membrane potential; Glutamic acid transport; Cation effect; (Rabbit jejunum vesicle)

We have applied our recently developed approach for quantitative generation and estimation of membrane potential differences (Berteloot, A. (1986) Biochim. Biophys. Acta 857, 180–188) to the reevaluation of glutamic acid transport rheogenicity in rabbit jejunal brush-border membrane vesicles. Membrane diffusion-potentials were created by altering iodide concentrations in the intra- and extravesicular compartments while keeping isosmolarity, isotonicity and ionic strength constant by chloride replacement. The known value of ion permeabilities relative to sodium in this preparation also allows calculation of membrane potential differences using the Goldman-Hodgkin-Katz equation. This strategy appears superior to more classical methods involving ionophore-induced membrane diffusion-potentials of protons or potassium as both cations have been shown to participate in the transport mechanism. In this paper, we demonstrate that this approach is perfectly suitable for the investigation of membrane potential dependency of glutamic acid transport as our results showed that chloride replacement by iodide did not affect uptake in vesicles with membrane potential clamped to zero by gramicidin D (sodium conditions) or by gramicidin D plus valimonycin (sodium + potassium conditions). The method thus allows to dissociate membrane potential effects from possible effects that might be introduced by altering the anion species. In these conditions, our studies clearly demonstrate that glutamic acid uptake, whether analyzed over a 1 min time scale or under initial rate conditions, was sensitive to membrane potential differences. However, our results also show that the electrogenicity of the transport system varied depending upon the intravesicular presence or absence of potassium, its presence stimulating the membrane potential dependency of uptake. This effect is modulated by the internal pH and it is concluded that inside H^+ and K^+ are not equivalent as countertransported cations. The external pH also seems to modulate the response to potential by acting on the fully loaded form(s) of the transporter. The possibility that outside H^+ competes for (an) external Na^+ binding site(s) and/or precludes the attachment of (an) extra sodium ion(s) should be considered.

Introduction

Our recent studies have determined some characteristics of glutamic acid transport by rabbit jejunal brush-border membrane vesicles [1]. It was shown that sodium is mandatory for uptake and that a sodium gradient (out > in) provides the driving force for uphill transport of the acidic amino acid, in accordance with other data on either intestinal [2,3] or renal [4–9] vesicles. It was also found that intravesicular potassium can stimulate the Na^+-dependent uptake with maximal effect under conditions of an outwardly oriented K^+-gradient [1], also in agreement with similar findings in intestinal [3] and renal [4,6–9]

Correspondence address: Membrane Transport Research Group, Department of Physiology, Faculty of Medicine, University of Montreal, C.P. 6208, succursale "A", Montreal, Quebec H3C 3T8 (Canada)

vesicles. Finally, it was determined that the K^+-effect is modulated by the surrounding pH and that a pH-gradient (acidic outside) can stimulate further both the Na^+- and the $Na^+ + K^+$-gradient dependent transport of L-glutamic acid [1], effects that were also reported for renal vesicles [7,8]. These results led to the proposal that the anionic amino acid carrier exists in both protonated and unprotonated forms inside the membrane. This conclusion is at variance with those proposed by Sacktor (OH^--L-glutamate exchange system) [7] and Nelson et al. (H^+ and K^+ competing for free transporter on both the internal and external surfaces of the membrane) [8] for the rabbit kidney brush-border membrane. From the models postulated by Berteloot [1] and Nelson et al. [8], one can predict that glutamate transport should be electrogenic, at least in some conditions. Moreover, if the suggestion that H^+ replaces K^+ as a countertransported ion [8] proves correct, it will render unlikely any change in electrogenicity when internal K^+ is removed. The determination of the membrane potential dependency of glutamic acid uptake thus appears crucial for the final interpretation of similar findings.

Conflicting results have been obtained in the past few years when trying to answer this question. Electrophysiological studies on rat renal proximal tubules in vivo [10] and guinea pig everted ileum in vitro [11] have demonstrated depolarisation of the brush-border membrane induced by acidic amino acid uptake. In contrast, in vitro studies with brush-border membrane vesicles isolated from rat small intestine [2] and rabbit [5,7] or rat [4] kidney have concluded to either an electroneutral [2,5,7] or an electrogenic [4] Na^+-dicarboxylic amino acid cotransport. Besides the possibility of species and/or organ differences, some technical problems are associated with these studies. Three different experimental strategies were used to determine whether Na^+-glutamate cotransport was electroneutral or electrogenic: (1) Membrane potential-sensitive optical probes [5,7]. (2) Generation of diffusion potentials by anions of different conductances [5,7]. (3) Generation of K^+ and H^+ diffusion potentials by specific ionophores, i.e. valinomycin and trifluoromethoxyphenylhydrazone (FCCP), respectively [2,5,7]. The dye approach is not very sensitive, has been associated with several possibilities for producing artefacts, and seems to give only semiquantitative results [12]. The second approach is mainly qualitative and variations in the ionic strength associated with anion replacement can affect the surface potential. The last approach suffers from the consideration that both K^+ and H^+, the two ions used to generate a membrane potential, are also substrates/products for the transport system and may serve as supplementary driving forces for active transport in gradient conditions [1,3,4,6–9]. Inhibitory effects resulting from the ionophore-induced dissipation of either the internal K^+-gradient or the external H^+-gradient could reduce or even mask any stimulation produced by the membrane potential generated in these conditions.

When looking closely at the published data using brush-border vesicles [4,5], it also seems that the conclusion as to rheogenicity of glutamic acid transport is dependent upon the time period during incubation over which the decision was made. The electrogenicity reported by Burckhardt et al. [4] was based on observations performed during the first 2 min of incubation. On the other hand, Schneider et al. [5] carried incubation for 90 min and concluded as to electroneutrality over the time period 5–20 min. It is apparent from Figs. 4–8 in Ref. 5 that the interpretation would have been different had these authors focused on observations up to 4 min.

It thus appears that vesicle studies should be reevaluated by using other approaches. In a recent report [13], we have justified the use of iodide as a highly permeant anion allowing quantitative generation of membrane diffusion-potentials in rabbit jejunal brush-border membrane vesicles. This paper describes experiments designed to reevaluate the electrogenicity of glutamic acid transport with this new method. Our results clearly demonstrate a transfer of charges during glutamic acid transport. However, the ionic environment and the surrounding pH determine the extent of the response to the membrane potential.

Materials and Methods

(1) Chemicals. All salts and chemcials for buffer preparation were of the highest purity available. D-[1(n)-^{3}H]mannitol (27.4 Ci/mmol) was from

New England Nuclear Corporation and L-[U-^{14}C]glutamic acid (290 mCi/mmol) was from Amersham. Amiloride hydrochloride was a gift from Merck, Sharp and Dohme Canada, Division of De Merck Frosst Canada Inc., Kirkland, Quebec. Ionophores, valinomycin and gramicidin D, were obtained from Sigma Chemical Company.

(2) Preparation of brush-border membrane vesicles. The small intestine was removed from 2.0–2.5 kg male New Zealand white rabbits (Ferme cunicole Leonard, Ste-Cholastique, Quebec) 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. [14] and vesicles were obtained by the method of Hopfer et al. [15] with slight modifications as described recently [1]. Based on sucrase activity, enrichment factors in the range 13–18-fold over the homogenate were routinely obtained.

(3) Transport studies. Uptake studies were carried out by the rapid filtration technique of Hopfer et al. [15] as described previously [1]. The freshly prepared vesicles were resuspended to a final protein concentration of 30–50 mg/ml with the final resuspension buffer and an aliquot (0.3–0.5 mg protein) was added to the incubation medium kept at room temperature (20°C) to start the transport experiments. The composition of the final resuspension buffers for vesicles and the final concentrations in the incubation media will be indicated in the legends of the figures. Amiloride was included in all transport experiments at a 0.5 mM concentration to get rid of any change that could occur through the Na^+/H^+ exchanger [13]. At time intervals, aliquots were taken from the incubation mixture, poured in 1 ml quenched ice-cold stop-solution as in Ref. 1, filtered on a prewetted and chilled 0.45 μm nitrocellulose filter (Satorius SM 11306) and washed with 4 ml of nonradioactive ice-cold stop-solution. Filters were then dissolved in mini-vials by 15 min incubation with 5 ml Filter Counter (United Technologies Packard) and subsequent vortexing. ^{3}H and ^{14}C radioactivities were determined using a Minaxi Tri-Carb Series 4000, model 4450 scintillation counter (United Technologies Packard).

Results are expressed as nmol solute uptake/mg protein. Membrane potentials, negative with respect to the vesicle interior, have been estimated using the Goldman-Hodgkin-Katz equation as described previously [13] and using the relative ion permeabilities reported for rabbit jejunal brush-border membrane vesicles [16]. Initial rates of transport, estimated by either single time point analysis (0.15 min uptake) or polynomial regression of the uptake time-curve [13,17], are expressed as nmol solute uptake/mg protein per min. Relative uptake values were calculated by normalisation to 100 when membrane potential was set to 0. Regression analysis have been performed using an Apple IIe microcomputer and a curve fitter program (P.K. Warme, Copyright © 1980, Interactive Microware Inc.). Statistical analysis were done with Statcalc [18] on the same microcomputer.

(4) Assays. Marker enzyme for the brush-border membrane, sucrase (EC 3.2.1.48), was routinely assayed by the method of Dahlqvist [19] as modified by Lloyd and Whelan [20]. Protein was measured according to Lowry et al. [21] with bovine serum albumin as standard.

Results

(1) Absence of iodide effects on glutamic acid transport

In order to use iodide-induced membrane potentials for generation of electrical potentials across the brush-border membrane vesicles according to our recently published approach [13], one must first justify the absence of iodide effects per se on glutamic acid uptake. This question was addressed in setting equal concentrations of Na^+ or Na^+ and K^+ on both sides of the vesicles and clamping the membrane potential to zero with gramicidin D or gramicidin D plus valinomycin, respectively. The results of this experiment are shown in Table I for sodium and in Table II for sodium plus potassium.

It first can be appreciated that glutamic acid transport was very fast in these conditions as equilibrium was reached at 0.75 min for all pH combinations tested with the exception of acidic outside pH gradients. However, different equilibrium values were sometimes obtained depending on the presence of chloride or iodide in the

TABLE I

GLUTAMIC ACID UPTAKE IN Na^+-CLAMPED CONDITIONS IN THE PRESENCE OF CHLORIDE OR IODIDE

Final resuspension buffers for vesicles: 50 mM Mes-Tris buffer (pH 6.0) or 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, and either 100 mM NaCl plus 100 mM choline chloride (anion Cl) or 100 mM NaI plus 100 mM choline iodide (anion I). Final concentrations in the incubation media (0.25 ml): 50 mM Mes-Tris buffer (pH 6.0) or 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, 0.05 mM L-[U-^{14}C]glutamic acid, 0.5 mM amiloride, 0.016 mg gramicidin D and either 100 mM NaCl plus 100 mM choline chloride (anion Cl) or 100 mM NaI plus 100 mM choline iodide (anion I). Values shown are the means ± S.D. of two experiments with different preparations of vesicles. One-way analysis of variance for each pH combination has been performed as described under Materials and Methods: [a] unsignificant difference ($P > 0.05$) as a function of time (row effect); [b] significant differences ($P < 0.05$) between iodide and chloride at a given time point (column effect); n.s., unsignificant differences between chloride and iodide at a given time point.

pH_{out}/pH_{in}	Anions	Glutamic acid uptake (nmol/mg protein) at different time intervals (min)			
		0.15	0.45	0.75	1.05
6/6	Cl	0.0451 ± 0.0055	0.0726 ± 0.0058	0.0871 ± 0.0020 [a]	0.0844 ± 0.0012 [a]
	I	0.0314 ± 0.0038 [b]	0.0638 ± 0.0054	0.0903 ± 0.0036 [a]	0.0934 ± 0.0057 [a]
			(n.s.)	(n.s.)	(n.s.)
8/8	Cl	0.0373 ± 0.0028	0.0758 ± 0.0058	0.1035 ± 0.0061 [a]	0.1150 ± 0.0062 [a]
	I	0.0362 ± 0.0076	0.0774 ± 0.0125	0.1128 ± 0.0043 [a]	0.1321 ± 0.0104 [a]
		(n.s.)	(n.s.)	(n.s.)	(n.s.)
6/8	Cl	0.0256 ± 0.0013	0.0656 ± 0.0042	0.1060 ± 0.0083	0.1260 ± 0.0066
	I	0.0225 ± 0.0035	0.0557 ± 0.0022	0.0905 ± 0.0060 [b]	0.1205 ± 0.0048
		(n.s.)	(n.s.)		(n.s.)
8/6	Cl	0.0489 ± 0.0045	0.0819 ± 0.0056 [a]	0.0862 ± 0.0065 [a]	0.0910 ± 0.0073 [a]
	I	0.0486 ± 0.0047	0.0866 ± 0.0045	0.1186 ± 0.0072 [ab]	0.1204 ± 0.0117 [ab]
		(n.s.)	(n.s.)		

TABLE II

GLUTAMIC ACID UPTAKE IN $Na^+ + K^+$-CLAMPED CONDITIONS IN THE PRESENCE OF CHLORIDE OR IODIDE

Final resuspension buffers for vesicles: 50 mM Mes-Tris buffer (pH 6.0) or 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, and either 100 mM NaCl plus 100 mM KCl (anion Cl) or 100 mM NaI plus 100 mM KI (anion I). Final concentrations in the incubation media (0.25 ml): 50 mM Mes-Tris buffer (pH 6.0) or 50 mM Tris-Hepes (pH 8.0), 0.1 mM $MgSO_4$, 0.05 mM L-[U-^{14}C]glutamic acid, 0.5 mM amiloride, 0.016 mg gramicidin D plus 0.003 mM valinomycin, and either 100 mM NaCl plus 100 mM KCl (anion Cl) or 100 mM NaI plus 100 mM KI (anion I). Values shown are the means ± S.D. of two experiments with different preparations of vesicles. n.s., [a] and [b] as in the legend of Table I.

pH_{out}/pH_{in}	Anions	Glutamic acid uptake (nmol/mg protein) at different time intervals (min)			
		0.15	0.45	0.75	1.05
6/6	Cl	0.0369 ± 0.0087	0.0733 ± 0.0095	0.0927 ± 0.0089 [a]	0.0928 ± 0.0048 [a]
	I	0.0268 ± 0.0030	0.0618 ± 0.0134	0.0896 ± 0.0080 [a]	0.1039 ± 0.0087 [a]
		(n.s.)	(n.s.)	(n.s.)	(n.s.)
8/8	Cl	0.0165 ± 0.0010	0.0309 ± 0.0025	0.0365 ± 0.0033 [a]	0.0396 ± 0.0020 [a]
	I	0.0162 ± 0.0013	0.0350 ± 0.0021	0.0463 ± 0.0024 [ab]	0.0503 ± 0.0019 [ab]
		(n.s.)	(n.s.)		
6/8	Cl	0.0168 ± 0.0010	0.0508 ± 0.0020	0.0798 ± 0.0064	0.0943 ± 0.0099
	I	0.0132 ± 0.0012	0.0408 ± 0.0025	0.0680 ± 0.0044 [b]	0.0941 ± 0.0034
		(n.s.)	(n.s.)		(n.s.)
8/6	Cl	0.0399 ± 0.0049 [a]	0.0355 ± 0.0045 [a]	0.0363 ± 0.0054 [a]	0.0319 ± 0.012 [a]
	I	0.0337 ± 0.0040	0.0570 ± 0.0108 [ab]	0.0561 ± 0.0085 [ab]	0.0489 ± 0.0081 [ab]
		(n.s.)			

medium. This behavior seems related to internal binding, as previously noted for equilibrium values in gradient conditions [1]. Next, Tables I and II show that 0.15 and 0.45 min uptake values were identical with either chloride or iodide as the only anion present in the incubation media, the only exception being at acidic pH conditions on both sides of the membrane with sodium as the cation (Table I). However, in this case, inspection of the drawn uptake curve indicates that the main effect of chloride was to increase the external binding component (intersection of the uptake curve with the y axis) while keeping the relative rates of uptake unaffected as compared to iodide. Finally, with sodium plus potassium present on both sides of the membrane (Table II), it should be noted that basic outside pH gradient conditions were unfavorable for uptake or that uptake was too fast in these conditions to be measured.

It thus seems that I^- and Cl^- behaved similarly as far as initial rates of glutamic acid transport were concerned and that this was true in both Na^+ and $Na^+ + K^+$ conditions, whatever the pH in the intra- and extravesicular compartments.

(2) Effect of membrane potential on glutamic acid uptake

The results presented in Fig. 1 have been obtained in Na^+- (out > in) + K^+- (in > out) gradient conditions when the pH was set at 8.0 on both sides of the vesicles and are representative of every situation in which membrane potential dependency was found. It first can be appreciated from Fig. 1 that membrane potentials negative with respect to the vesicle interior increased the rate of glutamic acid uptake while the opposite polarity decreased it. It also appears that going from inside negative to inside positive potentials changed the shape of the uptake curves. While linearity was observed up to 1.05 min at zero membrane potential, downward and upward curvatures were introduced by inside negative and inside positive membrane potentials, respectively.

Initial rates of glutamic acid uptake have been estimated from the data presented in Fig. 1 as described under Materials and Methods and plotted in Fig. 2 against the membrane potential difference across the brush-border membrane. It thus appears that initial rates of glutamic acid

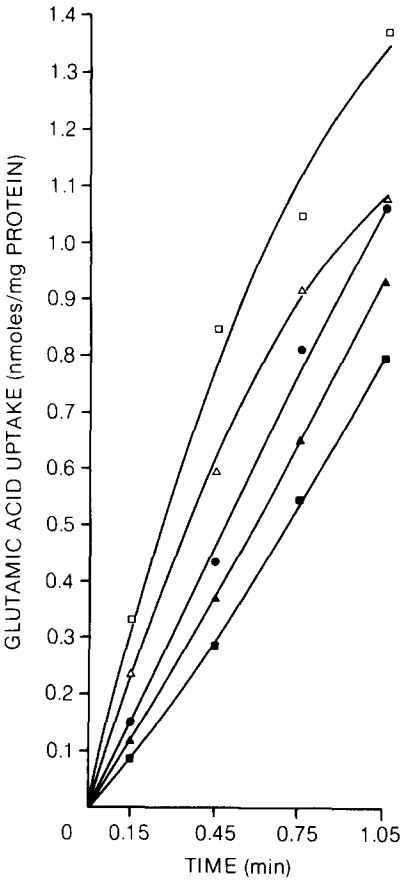

Fig. 1. Uptake time-course of glutamic acid uptake at different ratios of intra- to extravesicular iodide concentrations. Final resuspension buffers for vesicles: 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, 104 mM potassium iodide and/or chloride and 104 mM choline iodide and/or chloride to give iodide concentrations of 8 mM (□), 43 mM (△), 68 mM (not shown), 104 mM (●), 138 mM (not shown), 163 mM (▲), or 198 mM (■), respectively. Final concentrations in the incubation media: 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, 0.05 mM L-[U-^{14}C]-glutamic acid, 0.5 mM amiloride, 100 mM sodium iodide and/or chloride, 104 mM choline iodide and/or chloride and 4 mM potassium iodide and/or chloride to give iodide concentrations of 10 mM (■), 46 mM (▲), 71 mM (not shown), 104 mM (●), 140 mM (not shown), 165 mM (△) and 200 mM (□). Points shown are individual data points from the same preparation of vesicles and are representative of the five experiments performed in the same conditions. The drawn lines correspond to polynomial regression of the data points (coefficient of correlation higher than 0.99 in all cases).

uptake were exponentially related to the membrane potential amplitudes, whatever values were chosen for estimation of these initial rates. For this reason, we chose to use single time points as a

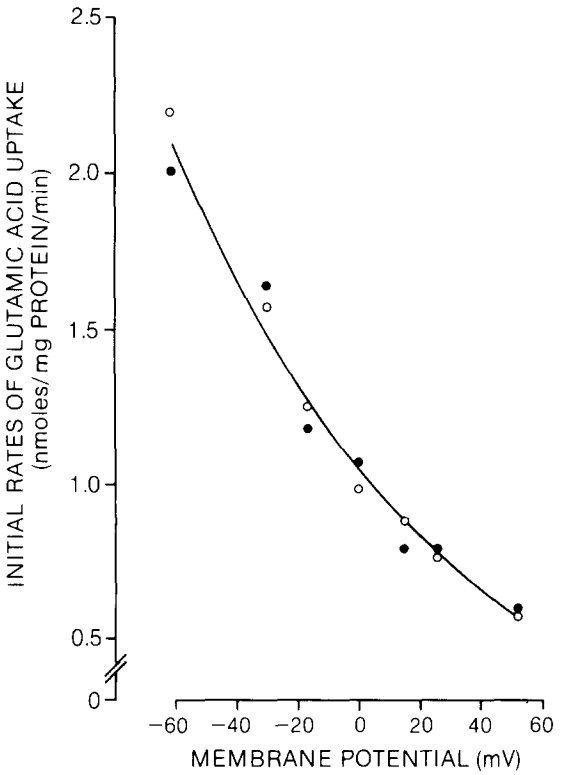

Fig. 2. Relationship between initial rates of glutamic acid uptake and membrane potential differences. Initial rates of glutamic acid uptake have been estimated from the data presented in Fig. 1 by polynomial regression (●) or by 10×0.1 min uptake values (○) as described in the text. Voltages negative with respect to the vesicle interior have been calculated using the Goldman-Hodgkin-Katz equation and relative ion permeabilities as reported in Ref. 16 (see text). Curve fitting has been performed as described under Materials and Methods. The drawn line corresponds to the equation: uptake = 1.0412 exp(0.01167 × membrane potential) with a coefficient of correlation of 0.9910.

reasonable approximation of initial rates in the following experiments. Fig. 2 clearly demonstrates that a potential difference, vesicle interior positive, inhibits sodium-coupled, potassium activated L-glutamic acid transport, while the opposite polarity promotes transport.

(3) Membrane potential dependency of glutamic acid transport in $Na^+ + K^+$-gradient conditions

Membrane potential dependency of glutamic acid transport in $Na^+ + K^+$-gradient conditions was analysed systematically with different pH combinations inside and outside the vesicles. Fig.

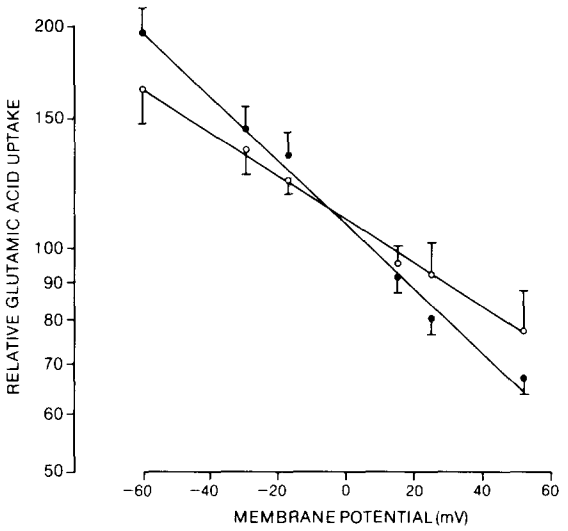

Fig. 3. Membrane potential dependency of glutamic acid uptake in $Na^+ + K^+$-gradient conditions at basic (●) and acidic (○) pH values. Final resuspension buffers for vesicles as in Fig. 1 with 50 mM Tris-Hepes buffer (pH 8.0) (●) or 50 mM Mes-Tris buffer (pH 6.0) (○). Final concentrations in the incubation media as in Fig. 1 with 50 mM Tris-Hepes buffer (pH 8.0) (●) or 50 mM Mes-Tris buffer (pH 6.0) (○). Points shown are the means ± S.D. of five experiments performed in duplicate on different preparations of vesicles ($n = 5$). Initial rates of glutamic uptake have been estimated by single (0.1 min) time point analysis. Voltages are as described in the legend of Fig. 2. Linear regression was performed as described in the text and gave y intercepts (0 potential), slopes, and coefficient of correlation of 110, $4.33 \cdot 10^{-3}$, 0.9986 (●) and 110, $2.99 \cdot 10^{-3}$, 0.9991 (○), respectively.

3 plots the log of relative initial rates of glutamic acid uptake versus membrane potential differences across the brush-border membrane at pH values of 6.0 and 8.0 on both sides of the membrane. Linear relationships were obtained, thus showing that electrogenic transport of glutamic acid uptake occurred at both pH values. However, the lower slopes recorded at the acidic pH clearly show that protons decreased the susceptibility of the transport system to membrane potential. Table III reports the results that were obtained in pH-gradient conditions. Again, electrogenicity of glutamic acid transport was demonstrated in these conditions.

(4) Membrane potential dependency of glutamic acid transport in Na^+-gradient conditions

From the data presented above, it appears that glutamic acid transport is electrogenic in the pres-

TABLE III

MEMBRANE POTENTIAL DEPENDENCY OF GLUTAMIC ACID UPTAKE IN $Na^+ + K^+$-GRADIENT CONDITIONS IN THE PRESENCE OF pH GRADIENTS

Final resuspension buffers for vesicles and incubation media as in the legend of Fig. 3. Values shown are the means ± S.D. of five experiments performed in duplicate on different preparations of vesicles ($n = 5$). Initial rates and membrane potentials have been determined as described in the legend of Fig. 3. One-way analysis of variance showed significant differences ($P < 0.05$) relative to −52 mV (a), −52, −33 and −16 mV (b), and all other values (c) for each column.

Membrane potential (mV)	Relative initial rates of glutamic acid uptake	
	pH_{out}/pH_{in} 6/8	pH_{out}/pH_{in} 8/6
−52	78 ± 4	73 ± 9
−26	85 ± 4	87 ± 10
−15	94 ± 4 [a]	94 ± 9
17	111 ± 9 [b]	111 ± 5 [a]
30	112 ± 8 [b]	124 ± 12 [b]
61	130 ± 11 [c]	181 ± 21 [c]

ence of both inward Na^+- and outward K^+-gradients as driving forces, in agreement with previous qualitative observations made by Bruckhardt et al.

TABLE IV

MEMBRANE POTENTIAL DEPENDENCY OF GLUTAMIC ACID UPTAKE IN Na^+-GRADIENT CONDITIONS AT ACIDIC pH AND IN THE PRESENCE OF pH GRADIENTS

Final resuspension buffers for vesicles and incubation media as in Fig. 4 except for buffers that were replaced by 50 mM Mes-Tris buffer (pH 6.0) when appropriate. Values shown are the means ± S.D. of four experiments performed in duplicate on different preparations of vesicles ($n = 4$). Initial rates and membrane potentials have been determined as described in the legend of Fig. 3. One-way analysis of variance showed significant differences ($P < 0.05$) relative to −61 mV (a) and −61, −33 and −16 mV (b) for each column.

Membrane potential (mV)	Relative initial rates of glutamic acid uptake		
	pH_{out}/pH_{in} 6/6	pH_{out}/pH_{in} 6/8	pH_{out}/pH_{in} 6/8
−61	102 ± 4	82 ± 6	94 ± 8
−33	100 ± 5	96 ± 7	101 ± 7
−16	106 ± 7	95 ± 8	100 ± 5
16	106 ± 7	95 ± 8	100 ± 5
16	106 ± 6	109 ± 7 [a]	115 ± 5 [a]
29	111 ± 10	112 ± 9 [a]	115 ± 9 [a]
60	110 ± 10	117 ± 10 [b]	127 ± 8 [b]

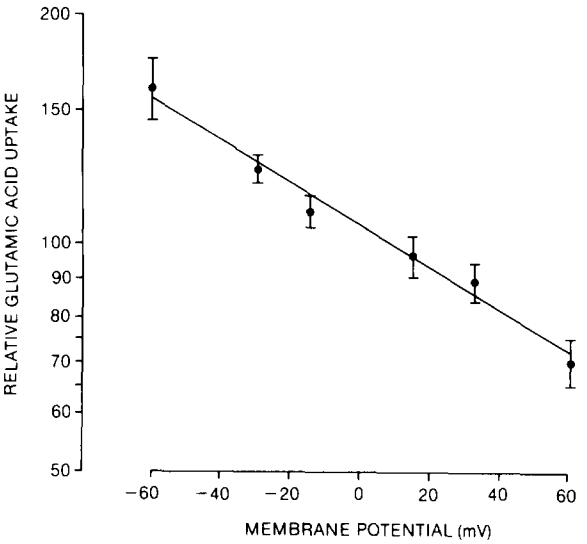

Fig. 4. Membrane potential dependency of glutamic acid uptake in Na^+-gradient conditions at basic pH. Final resuspension buffers for vesicles: 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$ and 208 mM choline iodide and/or chloride to get iodide concentrations shown in the legend of Fig. 1. Final concentrations in the incubation media: 50 mM Tris-Hepes buffer (pH 8.0), 0.1 mM $MgSO_4$, 0.05 mM L-[U-^{14}C]glutamic acid, 0.5 mM amiloride, 10 mM sodium iodide and/or chloride and 108 mM choline iodide and/or chloride to get iodide concentrations shown in the legend of Fig. 1. Points shown are the means ± S.D. of four experiments performed in duplicate on different preparations of vesicles ($n = 4$). Initial rates of glutamic acid uptake were estimated by single time point (0.15 min) analysis. Voltages are as described in the legend of Fig. 2. Linear regression was performed as described in the text and gave a y intercept of 105, a slope of $2.77 \cdot 10^{-3}$ and a coefficient of correlation of 0.9962.

[4] with rat renal brush-border membrane vesicles. As these authors also reported electroneutrality in Na^+-gradient conditions alone [4], we decided to investigate what would happen in our system under similar conditions. Table IV shows that glutamic acid transport was quite insensitive to membrane potential or weakly electrogenic with acidic conditions inside and outside the vesicles or pH gradient conditions, respectively. However, with basic pH conditions on both sides of the membrane, Fig. 4 clearly shows that glutamic acid transport was responsive to the membrane potential.

Discussion

In this paper, we have designed experiments aimed at reevaluating the rheogenicity of glutamic acid transport in brush-border membrane vesicles from rabbit small intestine. Our approach involved quantitative generation of membrane diffusion-potentials by altering iodide concentrations while keeping isosmolarity, isotonicity and ionic strength constant by chloride replacement [13]. This strategy has been validated for glucose transport and proved itself insensitive to inside potassium replacement by choline and to pH variations in the 6.0–8.0 range [13]. It thus appears perfectly suitable for our studies if it can be demonstrated that chloride replacement by iodide does not affect glutamic acid uptake.

The results presented in Tables I and II support this view as it was shown that glutamic acid uptake was identical in the presence of chloride and iodide in vesicles with membrane potential clamped to zero by gramicidin D (sodium conditions) or by gramicidin D plus valinomycin (sodium plus potassium conditions). However, it should be kept in mind that anions seem to influence glutamate binding in a pH-dependent way mainly on the internal side of the membrane under these conditions. This should not be a major problem in our studies as binding was shown to be a late event in gradient conditions [1], an observation confirmed by Fig. 1 as regression of the uptake curves goes through the origin. Our conclusion is also supported by the shape of the relation between glutamic acid uptake and membrane potential differences. Should the ions have influenced transport in anyway, one would have expected a Michaelian type of curve (or at least saturation) over the range of ion concentrations used in our studies (0 to 200 mM). This was obviously not the case (Fig. 2). It thus appears that our strategy for membrane potential generation should be successful in dissociating true membrane potential effects from possible effects that might be introduced by altering the anion species.

Using this new approach, our studies clearly demonstrate that glutamic acid uptake, whether analysed over a 1 min time scale (Fig. 1) or under initial rate conditions (Figs. 3, 4 and Tables II, III), was sensitive to membrane potential differences. These results agree with previous electrophysiological observations in vivo [10] and in vitro [11] showing membrane depolarization induced by acidic amino acid transport. Our results also agree with the electrogenicity of glutamic acid transport demonstrated for the L-glutamate transporter partially purified from pig renal brush-borders and reconstituted into proteoliposomes [22]. However, our studies represent the first attempt to correlate glutamic acid uptake and membrane potential differences.

In all cases where membrane potential dependency of glutamic acid transport was found, linear relationships between the log initial glutamate uptake and the membrane potential differences could be established, similar to those recently published for Na^+-glucose and Na^+-succinate cotransport systems in brush-border membrane vesicles from rabbit [13] and bovine [23] small intestines and from rabbit proximal tubules [24], respectively. The data presented in this paper are thus compatible with a relationship between membrane potential and glutamic acid uptake having the form

$$J = J_0 e^{K\Delta\psi}$$

where J is the initial rate of glutamic acid transport measured at different membrane potentials $\Delta\psi$, J_0 is the transport rate when $\Delta\psi = 0$ and K represents a constant that can be determined from the slope of straight lines analogous to those drawn in Figs. 3 and 4. Typical values for this constant have been determined by linear regression of the data presented in Figs. 3, 4 and Tables III, IV and are shown in Table V for comparison purposes. These values may be compared with $K \times 10^3 = 4.66$ for sodium-succinate cotransport in renal vesicles [24], and 1.55 or 8.16 for sodium-glucose cotransport in intestinal vesicles from bovine [23] or rabbit small intestine [13], respectively. However, as discussed previously for glucose transport [13], the above relation only corresponds to a very special case derived by Turner [25] and thus may not be valid anymore over a wider range of membrane potential differences or when varying the concentrations of the different effectors (Na^+, K^+, glutamic acid). Also, analyzing J over J_0 ratios at fixed effector concentrations as was done in our

studies puts the emphasis on the *K*-parameter in determining membrane potential dependency of transport. Assuming that steps perpendicular to the membrane plane (carrier mobility or reorientation) are the only ones susceptible to the membrane potential, it thus appears that *K* values are directly related to these steps but depend on several factors like the charge(s) being transferred from one side of the membrane to the other either during influx through (a) loaded complex(es) and/or during recycling of the transporter, and the point(s) in the electric field where the transition(s) of the carrier from form(s) *cis* to *trans* and/or *trans* to *cis* occur in the membrane [13,25].

With these restrictions in mind, the data summarized in Table V clearly show that the electrogenic character of glutamic acid transport was better expressed (highest values of K) in $Na^+ + K^+$-gradient conditions as compared to Na^+-gradient conditions alone for each set of pH combinations tested. Compared to other studies involving brush-border membrane vesicles, this result agrees with the qualitative observations made by Burckhardt et al. [4] but refutes the conclusions drawn by Corcelli et al. [2] and Schneider et al. [5]. As discussed in the Introduction, the contradiction may only be apparent as these last studies were not analysed in initial rate conditions. The stimulating effect of K^+ on membrane potential dependency of glutamic acid transport appears complementary to the previously proposed role for this cation, i.e. acceleration of (free) carrier recycling [1,8,9]. However, due to the complexity of the above-defined parameter *K*, it is not possible to ascribe the increased membrane potential sensitivity to either an increased transfer of positive and/or negative charge(s) through (a) fully loaded complex(es) and/or (a) recycling step(s), respectively, or a facilitation of (a) gating process(es), or any combination of these possibilities.

TABLE V

COMPARATIVE EFFECTS OF MEMBRANE POTENTIAL ON GLUTAMIC ACID UPTAKE IN DIFFERENT IONIC AND pH CONDITIONS

Data from Figs. 3 and 4 and Tables II and III have been analyzed as described in the text. Values shown are the means ± S.D. for *n* experiments in each cation-gradient condition.

Cation gradients	pH_{out}/pH_{in}	Coefficient of correlation	$K \times 10^3$
$Na^+ + K^+$ ($n = 5$)	6/6	0.9991	2.99 ± 0.10
	8/8	0.9986	4.33 ± 0.17
	6/8	0.9960	2.01 ± 0.13
	8/6	0.9916	3.29 ± 0.31
Na^+ ($n = 4$)	6/6	0.9309	0.35 ± 0.10
	8/8	0.9962	2.77 ± 0.17
	6/8	0.9850	1.26 ± 0.16
	8/6	0.9901	1.09 ± 0.11

Table V also shows that the electrogenic character of glutamic acid transport was greatly affected by inside and outside pH values either in the presence or absence of an outward K^+-gradient. However, differential responses were observed in both cases. In the absence of intravesicular K^+, membrane potential sensitivity of glutamic acid transport at different outside/inside pH combinations followed the potency order 6/6 < 8/6 < 6/8 < 8/8. It first appears that inside H^+ is preponderant over outside H^+ in determining the extent of the response to membrane potential. It also appears that outside H^+ decreases membrane potential dependency at fixed basic or acidic inside pH and that inside H^+ decreases it at fixed basic or acidic outside pH. Assuming that inside H^+ acts primarily on (the) recycling step(s) [1,8,9], these results could be compatible with inside H^+ decreasing the number of negative charge(s) flowing through the empty carrier(s) and/or the efficiency of the gating process(es) involved in recycling of the transporter. In this case, outside H^+ would primarily act by decreasing the number of positive charges flowing through the fully loaded complex(es) and/or the efficiency of the gating process(es) involved in reorientation of the(se) complex(es) inside the membrane.

In the presence of K^+, Table V shows that membrane potential sensitivity of glutamic acid transport at different outside/inside pH combinations followed the potency order 6/8 < 6/6 < 8/6 < 8/8. In this case, it appears that outside H^+ is preponderant over inside H^+ in determining the extent of the response to membrane potential. With K^+ accelerating (free) carrier recycling as previously proposed [1,8,9], such a result is not unexpected and would favor a role for outside H^+ as discussed above. However, the interactions be-

tween inside H^+ and K^+ (and possibly outside H^+) in determining potential sensitivity of glutamic transport appear more complex as opposite effects of inside H^+ are observed at fixed outside pH.

In conclusion, we have demonstrated that electrogenicity of glutamic acid transport depends on both the ionic environment and the surrounding pH. Inside potassium is necessary for the full expression of the rheogenic character and the effect is modulated by the intravesicular pH. These results rule out equivalent roles for intravesicular H^+ and K^+ as suggested by Nelson et al. [8]. The external pH also modulates the response to potential by likely acting on (a) step(s) involving the fully loaded form(s) of the transporter. The possibility that outside H^+ competes for (a) Na^+ binding site(s) and/or precludes the attachment of (a) extra sodium ion(s) should be considered.

Acknowledgements

The author was supported by a scholarship from the 'Fonds de la Recherche en Santé du Québec'. The technical assistance of Miss Lise Lessard has been greatly appreciated. The author thanks Miss Renée Chamberland for her secretarial help and Mrs. G. Filosi, D. Cyr and E. Rupnik for the art work. This study was supported by grant MA-7607 from the Medical Research Council of Canada.

References

1 Berteloot, A. (1984) Biochim. Biophys. Acta 775, 129–140
2 Corcelli, A., Prezioso, G., Palmieri, F. and Storelli, C. (1982) Biochim. Biophys. Acta 689, 97–105
3 Corcelli, A. and Storelli, C. (1983) Biochim. Biophys. Acta 732, 24–31
4 Burckhardt, G., Kinne, R., Stange, G. and Murer, H. (1980) Biochim. Biophys. Acta 599, 191–201
5 Schneider, E.G., Hammerman, M.R. and Sacktor, B. (1980) J. Biol. Chem. 255, 7650–7656
6 Schneider, E.G. and Sacktor, B. (1980) J. Biol. Chem. 255, 7645–7649
7 Sacktor, B. (1981) Mol. Cell. Biochem. 39, 239–251
8 Nelson, P.J., Dean, G.E., Aronson, P.S. and Rudnick, G. (1983) Biochemistry 22, 5459–5463
9 Fukuhara, Y. and Turner, R.J. (1985) Am. J. Physiol. 248, F869–875
10 Samarzija, I. and Fromter, E. (1982) Pflügers Arch. 393, 215–221
11 Himukai, M. (1984) Jpn. J. Physiol. 34, 815–826
12 Murer, H., Biber, J., Gmaj, P. and Stieger, B. (1984) Mol. Physiol. 6, 55–82
13 Berteloot, A. (1986) Biochim. Biophys. Acta 857, 180–188
14 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
15 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
16 Gunter, R.D., Schell, R.E. and Wright, E.M. (1984) J. Membrane Biol. 78, 119–127
17 Dorando, F.C. and Crane, R.K. (1984) Biochim. Biophys. Acta 772, 273–287
18 Lee, A.J., McInermey, P.J. and Mullins, P.R. (1984) Comput. Prog. Biomed. 18, 265–272
19 Dahlqvist, A. (1964) Anal. Biochem. 7, 18–25
20 Lloyd, J.B. and Whelan, W.J. (1969) Anal. Biochem. 30, 467–470
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
22 Keopsell, H., Korn, K., Ferguson, D., Menuhr, H., Ollig, D. and Haase, W. (1984) J. Biol. Chem. 259, 6548–6558
23 Kaunitz, J.D. and Wright, E.M. (1984) J. Membrane Biol. 79, 41–51
24 Wright, S.H., Hirayama, B.H., Kaunitz, J.D., Kippen, I. and Wright, E.M. (1983) J. Biol. Chem. 258, 5456–5462
25 Turner, R.J. (1981) Biochim. Biophys. Acta 649, 269–280