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Glutathione transport across intestinal brush-border membranes: effects of ions, pH, $\Delta\psi$, and inhibitors

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We characterized glutathione transport in brush-border membrane vesicles (BBMV) that were prepared from rabbit small intestine in which γ-glutamyl transpeptidases (γ-glutamyltransferases, EC 2.3.2.2) had been inactivated by a specific affinity-labeling reagent (AT_{125}). Intact GSH transport was strongly increased by the presence of Na^+, K^+, Li^+, Ca^{2+} and Mn^{2+} and, of all these, the Ca^{2+} activation effect was prevalent. This cation effect was selective and catalytic but not energetic; V_{max} obtained in the presence of both Na^+ and Ca^{2+} was about 6-times higher than it was in their absence, while K_m did not change. Moreover, these cations almost completely eliminated GSH binding on the membrane surface. Na^+ activation cannot be explained as a stimulation effect on the Na^+-H^+ antiport system, since a GSH proton-driven transport was excluded. We determined a pH optimum (7.5), while low or high extravesicular pH values diminished the GSH uptake rate. The Ca^{2+} effect on GSH transport, when an electrical potential difference was imposed across BBMV, was different from that of monovalent cations. Indeed, experiments performed by valinomycin-induced K^+ diffusion potential or by anion substitution showed that the GSH transport system was an electroneutral process in the presence of Na^+ or K^+, but that it was electrogenic in the presence of Ca^{2+} or in the absence of extravesicular cations. These results suggest that GSH is also cotransported with these cations, without its accumulation inside vesicles. Moreover, since GSH is negatively charged, the effect of pH changes and of cation activation on GSH transport is arguably mediated by changes in the ionization state of certain groups as the carrier site and of GSH itself, indicating the electrostatic nature of GSH binding sites on the transporter. The high Ca^{2+} activation effect is perhaps also partly due to fluidity changes in the lipoproteic microenvironment of the GSH transporter. Moreover, this transport system has high affinity with GSH, given the low K_m value (17 μM) and the fact that it was only inhibited by GSH S-derivatives and by GSH monoethyl ester, which probably share the same transport system.

Introduction

The epithelium of the small intestine is directly exposed to a number of drugs, food, additives and xenobiotics. It represents a first line of defence against various infested toxic chemicals. It is known that intracellular glutathione is an important compound in the detoxication process and in cellular protection against chemical injury [1–5] in various tissues. The liver releases GSH at a substantial rate and helps maintain the circulation of GSH in the plasma [6,7]. Nevertheless, it has recently been demonstrated that the uptake of exogenous intact GSH provided rat small-intestinal and renal epithelial cells with significant protection against chemically induced injuries [8,9]. Moreover, the presence of GSH transport systems in both poles of the intestinal cells (brush-border and baso-lateral membranes) [10,11], may supply GSH to plasma and to other cells where it may aid cellular detoxication [11].

This suggests the validity of the oral therapeutic administration of GSH under the various physiological and pathological conditions that cause a lack of endogenous GSH. For this reason we characterized the Na^+-independent transport system of GSH in rabbit intestinal BBMV. A previous paper of ours [10] provided evidence that intact GSH transport exists in these membranes, whose γ-glutamyltranspeptidase (EC 2.3.2.2) activity was inactivated by a specific affinity-labeling agent

Abbreviations: GSH, glutathione; AT_{125}, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; BBMV, brush-border membrane vesicles.

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AT_{125}. The present study was partly prompted by the scarce and incomplete understanding of intestinal peptide transport [12,13] particularly concerning the ion and energy-dependence and the specificity of transport. To clarify these aspects, we studied the effects of mono and bivalent cations and of proton gradients on GSH uptake. Moreover, since at physiological pH GSH is the only negatively charged tripeptide whose specific systems of transmembrane transport had already been demonstrated [8,10,14–16], we investigated the effect of membrane potential on GSH uptake to test whether it is an electrogenic or an electroneutral system. We also determined the kinetics and the specificity of this GSH transporter by using the inhibition effect of the γ-glutamyl compounds, GSH analogues and Probenecid (an inhibitor of Na^+-dependent GSH transporter) [8].

Materials and Methods

Materials

[*glycine*-2-^{3}H]GSH (spec. activity 1 Ci/mmol), was obtained from New England Nuclear (Boston, MA). D-[U-^{14}C]Glucose, from Amersham Radiochemical Center, Ltd., Bucks. AT_{125} was a gift from Upjohn Company (Kalamazoo, Michigan). Glutathione monoethyl ester was obtained by the procedure of Bergman and Zervas [17]. Nitrocellulose filters (0.45 μm pore size) came from Sartorius (Göttingen, F.R.G.). The dye-reagent concentrate for the determination of protein was obtained from Bio-Rad. All other chemicals used were reagent grade and were obtained from commercial sources.

Preparation of brush-border membrane vesicles

BBMV were prepared from frozen rabbit small intestine (jejunal and ileal segments) by the method described by Schmitz et al. [18] and modified by Kessler et al. [19]. After final centrifugation, the membranes were suspended in a medium containing 300 mM mannitol, 0.1 mM $MgSO_4$, 10 mM Tris-Hepes (pH 7.5) and 1.5 mM DTT. Protein concentration was about 15 mg/ml, as determined by Bredford's method [20]. The following enzymes were assayed as markers for the brush-border membrane: alkaline phsphatase, leucine aminopeptidase, γ-glutamyltranspeptidase and sucrase. The specific activities of these enzymes were enriched 16-, 10-, 8-, and 11-fold, respectively, compared to that of intestinal crude homogenate. The activity of lactate dehydrogenase, a cytosolic enzyme, was reduced 70-fold and the activity Na^+/K^+-ATPase, a basolateral membrane marker enzyme, was absent in the vesicles. Moreover, microscopic examination revealed predominantly unilamellar membrane vesicles.

Transport measurements

Before the uptake experiments, the γ-glutamyltranspeptidase activity in BBMV was completely inactivated by incubation at 30 °C for 30 min with a 0.5 mM AT_{125}, an affinity labeling reagent for glutamine amide transferases; as previously reported [10], this reagent under these experimental conditions does not alter membrane functionality.

The uptake of radiolabeled substrates into intestinal BBMV was performed by a rapid filtration method, as described by Hopfer et al. [21]. Deaerated solutions under nitrogen were used for transport studies. In a final volume of 30 μl the transport media contained 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5) and 0.5 mM DTT, varying concentrations of the radioactive unlabeled substrates, ions and mannitol to adjust medium osmolality.

Transport was started by adding AT_{125} treated BBMV (150–200 μg protein). The final concentration of AT_{125} was 0.16 mM. After appropriate incubation times (from 5 s to 60 min), transport was terminated by the addition of 3 ml ice-cold 0.15 M NaCl buffered with 1 mM Hepes-Tris pH 7 'stop solution'. The mixture was then quickly filtered through a nitrocellulose filter and washed with 3 + 3 ml 'stop solution'. Vesicle-associated radioactivity on the filters was determined in a liquid scintillation counter. All values were corrected for radioactivity found on those filters that lacked membrane vesicles. Washing membrane vesicles with a hypotonic solution eliminated most of the intravesicularly transported free-form ligands [22]. Thus, after equilibrium uptake, the material inside the vesicles on the filters was eluted with water and concentrated by lyophilization to a small volume. In this concentrated solution, GSH was measured by the glutathione reductase method, using unlabeled GSH (10–200 ng/ml of assay mixture) as the standard [23]. This enzymic analysis revealed that about 90% of the radioactivity associated with AT_{125}-treated vesicles was due to intact GSH, as also reported by Inoue et al. [14] in renal BBMV.

In experiments in which the effect of membrane potential on GSH transport was studied, vesicles were pre-equilibrated with valinomycin (15 μg/mg protein), K^+ ionophore [24] and subsequently exposed to different intravesicular/extravesicular K^+ ratios. In the present study 'interior negative', as opposed to the extravesicular side, was achieved via 20-fold dilution into a K^+-free uptake buffer of membrane vesicles preequilibrated in 100 mM KCl. The presence or absence of valinomycin then served for the formation of either a negative membrane potential or the reference control condition. Likewise 'interior positive' was achieved via an inwardly directed K^+ gradient in the presence of valinomycin. For some experiments, the membrane potential was 'clamped' at 0 mV by the addition of valinomycin to membrane vesicles with equimolar K^+ concentrations in intra- and extravesicular solutions.

This procedure has already been demonstrated as effectively short circuiting membrane potential [25].

Valinomycin was added in ethanol; the final concentrations of ethanol were 0.5 and 0.25%. Control measurements showed that these concentrations of ethanol and valinomycin in the absence of K^+ had no effect on GSH transport into BBMV.

There were small variations in transport activity. With differing membrane preparations the results agreed to within 10%; however, all experiments were repeated at least three times, each experiment always being triplicated. The reported data represent means ± S.D., when the bar is very small it is not indicated. Further experimental conditions are described in the figure legends.

Results

Effect of mono- and bi-valent cations on GSH uptake

The data reported in the literature about the effect of Na^+ or intestinal GSH transport are contradictory, probably because the experiments were performed using different animal species and different techniques: pig intestinal BBMV [26]; rat intestinal everted sac [27] (in both studies the γ-glutamyltranspeptidase activity was not suppressed by AT_{125}); and vascularly perfused rat small intestine [11]. For this reason we studied the effect of Na^+ and of other mono- and bi-valent cations (in the form of chloride salts) on the rate of GSH transport in AT_{125}-treated BBMV in which GSH hydrolysis and the cellular metabolic event were eliminated. Table I shows the lack of specificity in monovalent cations (Na^+, K^+, Li^+), all of which increase GSH uptake rate by about 50%, whereas in the bivalent cations the activation effect of Ca^{2+} and Mn^{2+} prevails, and is higher than the effect of monovalent cations, considering that Ca^{2+} and Mn^{2+} are present at a concentration that is 20-times lower. Moreover, an inward-gradient of $choline^+$ or $Tris^+$ or Mg^{2+} does not increase GSH uptake, indicating that the activation effect was not only due to the positive charge (one or two) but to the presence of the cation *per se*. Uptake equilibrium values are similar and this shows that extravesicular ions had no effect on the intravesicular volume. The reported concentrations are those in which we obtained maximum GSH uptake values. These results are confirmed by the fact that the same GSH uptake time course behaviour is obtained in the presence of $choline^+$ gradient or in the absence of cations (Fig. 1). By contrast, in the presence of extravesicular Na^+ or K^+ or Ca^{2+} gradients, the time courses were mutually similar, and GSH uptake values were higher. We therefore directed our attention to the activation effect of Na^+ or Ca^{2+} on the GSH transport system. Table II shows that the percentage of activation obtained in the presence of an inward Na^+ or Ca^{2+} gradient on the initial rate of GSH uptake (after 10 s of incubation) is similar to that obtained after 1 min of incubation and that at the same concentration (5 mM) the Ca^{2+} activation effect is three times higher than that obtained with Na^+. Moreover, in the presence of the same cation concentration on both sides of the membrane vesicles (100 mM out, 100 mM in, for Na^+; and 5 mM out, 5 mM in, for Ca^{2+}) the GSH uptake values are similar to those obtained when an inward-gradient

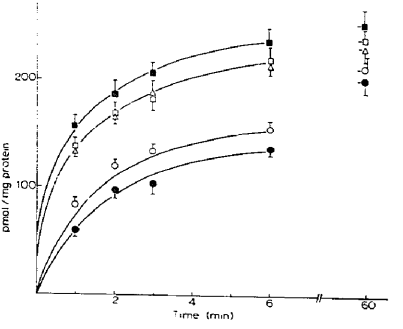

Fig. 1. Time course of GSH uptake in the presence of inward cation gradients. Transport was carried out with vesicles (30 μl) in uptake media containing: 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH and: 5 mM $CaCl_2$, 285 mM mannitol (■) or 100 mM KCl, 100 mM mannitol (Δ) or 100 mM NaCl, 100 mM mannitol (□) 100 mM choline chloride, 100 mM mannitol (●) or 300 mM mannitol (○).

TABLE I

Effect of mono- and bi-valent cations on GSH uptake into intestinal BBMV

The cations (all as chlorides) are present in the extravesicular media. Transport was carried out with BBMV (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH and 100 mM Na^+, K^+, Li^+, $choline^+$, $Tris^+$, or 5 mM Ca^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+} and 185 mM mannitol or only 200 mM mannitol to maintain osmolarity. The values in parenthesis are the activation % of GSH uptake compared to the control (without cations in the uptake media). The data represent means ± S.D. for five preparations. * $P < 0.005$ and ** $P < 0.05$ compared with the control value.

Addition	Uptake (pmol GSH/mg protein)	
Incubation:	1 min	60 min
None	85 ± 9	220 ± 30
Na^+	140 ± 8 (60) *	230 ± 15
K^+	130 ± 10 (50) *	200 ± 20
Li^+	120 ± 15 (40) *	210 ± 10
Ca^{2+}	145 ± 16 (70) *	250 ± 25
Cd^{2+}	100 ± 10 (17) **	230 ± 30
Mn^{2+}	120 ± 15 (40) *	200 ± 15
Mg^{2+}	90 ± 10 (0)	210 ± 20
$Choline^+$	80 ± 10 (0)	210 ± 20
$Tris^+$	90 ± 12 (0)	230 ± 25

TABLE II

Effect of differing intra and extravesicular concentrations of Na^+ and Ca^{2+} on GSH uptake into intestinal BBMV

Transport was carried out with BBMV (30 μl) in uptake media containing 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH and various concentrations of Na^+ and Ca^{2+} and various mannitol concentrations to maintain osmolarity. ° Vesicles were preloaded for 1 h with Na^+ or Ca^{2+} solutions (as chlorides) to obtain final concentrations (out = in) of 100 mM and 5 mM, respectively. The values in parentheses are the activation % of GSH uptake compared to the control (without cations in the uptake media). The data represent means ± S.D. for five preparations. * $P < 0.005$ and ** $P < 0.01$ compared with control value.

Addition	Uptake (pmol GSH/mg protein)	
Incubation:	10 s	60 s
None	40 ± 5	90 ± 9
Na^+ (5 mM out/0 in)	50 ± 4 (25) **	115 ± 9 (27) *
Na^+ (100 mM out/0 in)	60 ± 8 (50) *	140 ± 15 (55) *
°Na^+ (100 mM out/100 mM in)	–	150 ± 11 (66) *
Ca^{2+} (0.5 mM out/0 in)	48 ± 3 (20) **	120 ± 10 (33) *
Ca^{2+} (5 mM out/0 in)	70 ± 5 (75) *	155 ± 14 (70) *
°Ca^{2+} (5 mM out/5 mM in)	–	160 ± 15 (77) *

(100 mM out, 0 in, for Na^+; 5 mM out, 0 in, for Ca^{2+}) is present, indicating that the increase of GSH uptake is fundamentally related not to the inward cation gradient but to the cation concentration *per se*. We had previously demonstrated that with an inward Na^+ gradient, GSH binding to membrane surfaces is only 6% [10]. We therefore performed the same experiments with a Ca^{2+} extravesicular concentration of 5 mM to test whether the higher GSH uptake values obtained with Ca^{2+} truly reflected GSH transmembrane movement. The results reported in Fig. 2 show that GSH binding is about 8.5% ± 2.5 in the presence of Ca^{2+} while in the absence of Ca^{2+} it is about 21% ± 4.5. Therefore, in this case, vesicle-associated radioactivity represents GSH transmembrane transport into an osmotically active space.

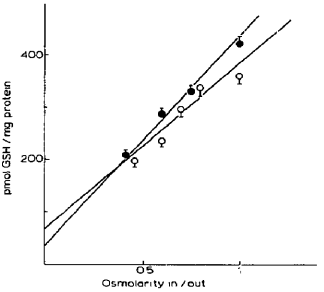

Fig. 2. Effect of osmolarity on the equilibrated GSH content of membrane vesicles. Transport was carried out with membrane vesicles (30 μl) in uptake media containing: 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH varying concentrations of mannitol (300–700 mM) in the absence (○) and in the presence (●) of 5 mM $CaCl_2$. GSH uptake values were determined after 60 min of incubation. The ratios for osmolarity inside/osmolarity outside the vesicles are reported on the abscissa. The intercept that is greater than 0 shows that binding of GSH to the vesicles is 8.5% in the presence of Ca^{2+} and 21% in its absence.

Effect of proton gradient on GSH uptake

Recently, Ganapaty and other authors [13,28,29] reported that an inward proton gradient stimulated and energized the transport of certain peptides. Moreover, the partial dependence of peptide uptake on Na^+ in intact tissue preparation was explained as an inward Na^+ gradient stimulation effect on the Na^+-H^+ antiport system [13,30]. We examined the effect of different extravesicular pH values (pH_o) on GSH uptake. Fig. 3 shows that GSH uptake was enhanced neither by an inward nor by an outward proton gradient. Moreover, it is evident at varying incubation times that GSH uptake is significantly higher when pH_o and pH_{in} (intravesicular) are 7.5. This seems to be the optimal pH for GSH uptake, and every uptake experiment was performed at this pH value. Uptake equilibrium values are all similar, suggesting that the various extravesicular pH values had no effect on intravesicular volume. These results show that the GSH uptake process is not proton-driven. Moreover, the addition of protonophore FCCP to the uptake media (when pH_o was 5.5 and pH_{in} 7.5) had no effect on GSH transport, indicating that it was not the H^+ gradient which decreased the GSH uptake rates, but

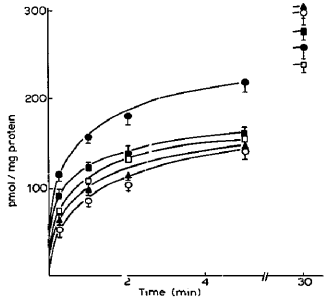

Fig. 3. Effect of extravesicular pH on GSH uptake. Transport was carried out with membrane vesicles (30 μl) in uptake media containing: 300 mM mannitol, 15 μM [^{3}H]GSH, 35 μM unlabeled GSH, 0.5% ethanol and 10 mM Hepes-Tris pH_o 7.5 (●) or pH_o 6 (■) or pH_o 5.5 (○) or pH_o 5.5 + 90 μM FCCP dissolved in ethanol 0.5% (▲) or pH_o 9 (□). pH_{in} was always 7.5. The values are the means of three experiments performed in triplicate ± S.D.

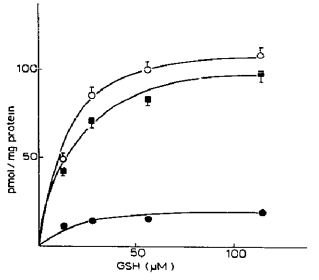

Fig. 4. Effect of varying GSH concentration on the initial rate of GSH uptake. GSH uptake values for mediated transport were obtained by subtracting the diffusion component for each concentration after 10 s of incubation with membrane vesicles (30 μl) in uptake media containing: 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), varying concentrations of [^{3}H]/GSH (14–130 μM) and: 100 mM choline chloride (●) or 100 mM NaCl (■) or 5 mM $CaCl_2$ and 185 mM mannitol (○).

the low outside pH *per se*. The same results were obtained when the experiments were performed in the presence of an inward Na^+ or K^+ or Ca^{2+} gradient and the maximum values of GSH uptake were always at pH_o 7.5 = pH_{in} (data not shown).

Kinetic data of GSH uptake

To investigate whether the action of Ca^{2+} differs from that of other cations on the GSH transport system, we studied the effects of varying GSH concentrations on the initial rate (after 10 s of incubation) of GSH uptake in the presence of extravesicular Ca^{2+} or Na^+ or choline$^+$ gradients (Fig. 4); as previously determined, GSH uptake is linear at up to 10 s of incubation [10]. The uptake values at the various GSH concentrations were then corrected for diffusion by subtracting each concentration's diffusion component to yield the net transport mediated uptake; the diffusional component was obtained multiplying the slope of the linear part of the curve by each solute concentration as reported in a previous work [10]. The initial rate of GSH transport followed Michaelis-Menten saturated kinetics. The behaviour is hyperbolic and the reciprocal plots obtained with these data are linear (not shown). However, the activation effect of Na^+ or Ca^{2+} on initial GSH uptake is evident and is similar for both. This greater capacity in the transport system is confirmed by V_{max} values (Table III), which are six times higher than in the presence of choline$^+$, while the affinity for GSH does not change; indeed, K_m values are all similar. We also determined GSH uptake values in the presence of varying extravesicular Ca^{2+} or Na^+ concentrations after 10 s of incubation. In both cases, the time course

TABLE III

Comparison of the kinetic parameters of GSH transport in the presence of an inward cation gradient

The value ± S.D. were obtained from a reciprocal plot of the data reported in Fig. 4.

Addition	K_m (μM)	V_{max} (pmol GSH/mg protein per 10 s)
Choline$^+$	17 ± 3	20 ± 5
Na^+	23 ± 3	120 ± 10
Ca^{2+}	25 ± 4	130 ± 8

behaviour was hyperbolic, indicating that this activation effect was not allosteric (Fig. 5).

Effect of membrane potential on GSH uptake

The potential difference across biomembranes represents an important driving force for electrogenic solute transport [24]. Since GSH is negatively charged at physiological pH, the anionic nature of GSH may contribute to its own transmembrane movement. To test this hypothesis and to determine whether the studied cations were associated with the GSH transport system and were translocated with GSH, the effect of an artificially imposed membrane potential difference on transport activity was studied, using two methods: (1) anion substitution and (2) valinomycin-induced K^+ diffusion potential. In the first experiment the effect of Na^+ salts with differing anion permeabilities ($SCN^- > Cl^- > SO_4^{2-}$) was tested on GSH uptake. Since NaSCN enters the vesicles faster, a negative membrane potentialis generated earlier than with Na_2SO_4 or NaCl. Table IV depicts that after 1 and 2 min of incubation in the presence of the three Na^+ salts, there was no significant difference in GSH uptake values, indicating an electroneutral process. Thus the negative charge of GSH is balanced by the simultaneous cotransport of cations or

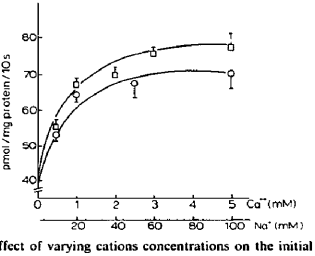

Fig. 5. Effect of varying cations concentrations on the initial rate of GSH uptake. Transport was carried out with membrane vesicles (30 μl) in uptake media containing 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH, varying mannitol concentrations to maintain osmolarity and varying Ca^{2+} (□) or Na^+ (○) concentrations.

TABLE IV

Effect of negative membrane potential induced by anions on GSH uptake

Transport was carried out with BBMV (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GSH and 100 mM NaSCN or 100 mM NaCl or 50 mM Na_2SO_4, and 50 mM mannitol. The data represent means ± S.D. for triplicates.

Incubation time (min)	Uptake (pmol GSH/mg protein)		
	NaSCN	NaCl	Na_2SO_4
1	130 ± 10	140 ± 8	150 ± 10
2	170 ± 15	180 ± 12	175 ± 15

by the simultaneous antiport of anions. This result was confirmed by experiments that involved a valinomycin induced K^+ diffusion potential (interior positive). Fig. 6 does not show changes in values for GSH uptake into vesicles in the presence of valinomycin, just as in voltage-clamp conditions (valinomycin and $[K^+]_{in} = [K^+]_{out}$), the time course behaviour is similar. In these experiments in the extravesicular media, Na^+ or K^+ cations are always present. To confirm these data we performed experiments that involved an interior negative membrane potential induced by valinomycin in the presence of an outward K^+ gradient. In this case, we obtained data that contrasted with those of previous experiments. In the absence of extravesicular cations or in the presence of extravesicular Ca^{2+}, the addition of valinomycin (interior negative) reduced GSH uptake into vesicles (Fig. 7). These transport profiles are characteristic of a membrane potential-dependent transport system, indicating an electrogenic process. On the contrary, the simultaneous presence of a negative membrane potential and an inward Na^+ gradient did not affect GSH uptake (Fig. 7). In conclusion, the experiments with anion replacement and valinomycin-induced K^+ diffusion potential (in which there are Na^+ or K^+ in the extravesicular media), suggest that the GSH transport system is electroneutral. By contrast, in the presence of interior negative, when there are no extravesicular cations (Na^+, K^+) or when Ca^{2+} is present, the GSH transport process is electrogenic (with a negative charge transfer). By way of control, experiments with K^+ diffusion potential generated by valinomycin were performed on Na^+ glucose transport. As was predictable from the electrogenic nature of its transport, D-glucose uptake increased several-fold under interior negative membrane potential conditions, while it decreased in the presence of positive interior or of voltage-clamp conditions (not shown).

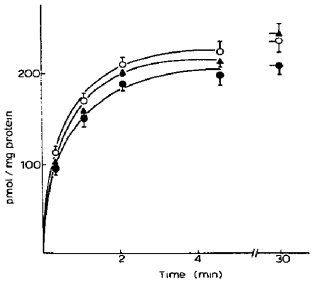

Fig. 6. Effect of K^+ diffusion potential (interior positive) generated by valinomycin on GSH uptake. Transport was carried out with membrane vesicles (30 μl) in uptake media containing: 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 15 μM [^{3}H]GSH, 35 μM unlabeled GsH, 100 mM KCl, 0.5% ethanol (○) or valinomycin (15 μg/mg protein) (●). Membrane vesicles were also incubated with valinomycin and 100 mM KCl for 20 min to eliminate any ion gradient across the membrane (voltage-clamp conditions) (▲).

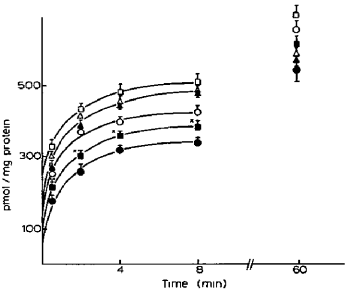

Fig. 7. Effect on GSH uptake of a simultaneous presence of K^+ diffusion potential (interior negative) generated by valinomycin and extravesicular cations. Membrane vesicles were prepared in a medium containing 100 mM mannitol, 100 mM KCl, 10 mM Hepes-Tris (pH 7.5) and an aliquot was mixed with uptake media with a final composition of: 10 mM Hepes-Tris (pH 7.5), 5 mM KCl, 30 μM [^{3}H]GSH, 45 μM unlabeled GSH and: 100 mM choline chloride, 90 mM mannitol (○, ●) or 100 mM NaCl, 90 mM mannitol (△, ▲) or 5 mM $CaCl_2$, 275 mM mannitol (□, ■). Vesicles with valinomycin (15 μg/ml protein) (closed symbols) or with 0.25% ethanol alone (open symbols). The data represent the means of four experiments ± S.D. * $P \leq 0.01$, ** $P < 0.001$ compared with values obtained without valinomycin.

Effect of probenecid, ophthalmic acid, GSH ethyl ester, γ-glutamyl compounds and GSH S-derivates on GSH uptake

To characterize the intestinal brush-border GSH transporter in comparison to those determined in other membranes [8,14–16], we studied the effect of varying compounds on the time course of GSH transport. The results reported in Fig. 8 indicate that GSH transport was unaffected when probenecid or ophthalmic acid (a

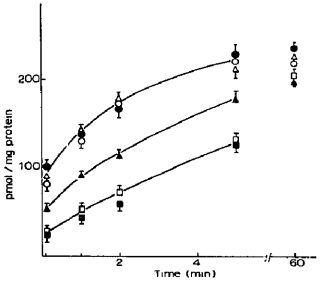

Fig. 8. Effect of probenecid, ophthalmic acid and glutathione derivatives on GSH uptake. Transport was carried out with membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 100 mM NaCl, 15 μM [^{3}H]GSH, 35 μM unlabeled GSH (△) and the presence of: 0.5 mM probenecid (●) or ophthalmic acid (γ-Glu-α-aminoisobutyrate-Gly) (○) or *S*-ethyl-GSH (▲) or *S*-*p*-nitrobenzyl-GSH (■) or γ-Glu-Cys-Gly ethyl ester (□).

GSH analogue which possesses α-aminoisobutyrate substituted for cysteine) were present in uptake media at a concentration 10-times higher than that of GSH, while *S*-ethyl-GSH, *S*-*p*-nitrobenzyl-GSH and GSH ethyl ester (γ-glutamylcysteinyl glycyl ester) reduced the rate of GSH uptake into the vesicles by about 30%, 60% and 65%, respectively, at the shortest incubation times (10 s, 1 min). Moreover, we performed inhibition experiments on GSH transport, using other γ-glutamyl compounds, such as γ-glutamylglutamate, γ-glutamyl-*p*-nitroanilide,

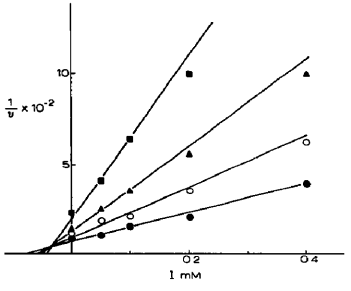

Fig. 9. Dixon Plot. Competitive inhibition of GSH uptake by GSH ethyl ester. Transport was carried out with membrane vesicles (30 μl) in uptake media containing 100 mM mannitol, 10 mM Hepes-Tris (pH 7.5), 100 mM NaCl, increasing inhibitor concentrations (0.05–0.4 mM) and: 13 μM [^{3}H]GSH (■) or 27 μM [^{3}H]GSH (▲) or 54 μM [^{3}H] GSH (○) or 110 μM [^{3}H]GSH (●). *V* = pmol GSH taken up per mg protein after 10 s of incubation. I, GSH ethyl ester.

γ-glutamylglycine and γ-ethylglutamate. None of these compounds decreased GSH uptake (data not shown).

We also carried out a kinetic analysis of GSH monoethyl ester inhibition at the initial rate of GSH uptake (10 s). This GSH derivative behaves as a competitive inhibitor against GSH uptake when presented in a Dixon plot (Fig. 9); the same results were obtained from Lineweaver-Burk and from Hunter-Down plots. Furthermore, in this experiment the diffusional component was subtracted from uptake values obtained at the varying GSH concentrations, both in the presence and in the absence of an inhibitor. The apparent K_i mean value for GSH uptake is 33 μM.

Discussion

The literature reports contradictory and scarce data on the characteristics of specific transporters of intac peptides across plasma membranes and on the question of ion-dependence. This is due in part to differences in technique and in part to the hydrolysis of the peptides on the membrane surface. Nevertheless, there is now adequately sound evidence that peptides, including certain biologically active ones, can be and are absorbed across small intestine in intact form. For example, the biological efficacy of LH-RF (luteinizing-hormone releasing factor) and of TRF (thyrotropin-releasing factor) were proved when administered orally [12].

In a previous paper [10], we demonstrated that intact GSH enters AT_{125}-treated vesicles obtained from intestinal brush-border membrane, and Hagen has demonstrated that the degradation of GSH and the transport of intact GSH are mutually independent and occur simultaneously [11].

Until now, in the intestinal membrane, the Na^+-dependent, Na^+-independent and proton dependent transport systems of varying peptides have been identified: there are no data, however, on the effect of other cations on transmembrane transporters. There are only reports for a Ca^{2+} activation effect on glucose Na^+-dependent transport [31] and on GSH transport in the liver sinusoidal plasma membrane [32].

In the present study we have clearly demonstrated that facilitated GSH transport is strongly affected by the presence of Na^+, K^+, Li^+, Ca^{2+} and Mn^{2+}, which increase GSH uptake into vesicles several-fold and that, of all these, the Ca^{2+} activation effect is prevalent. This effect is neither due to the positive charge alone (be it mono or bivalent) nor to a cation gradient, but to the presence of certain cations. Thus we can confirm that GSH is translocated by Na^+-independent transport and that the cation effect is selective and catalytic but not energetic. This is confirmed by kinetic data; V_{max} obtained in the presence of Na^+ or Ca^{2+} is about 6-times higher than in their respective absences while GSH affinity for the transporter does not change. These

cations accelerate the equilibration of GSH without enabling accumulation.

Na^+ activation can explain what was reported by Hagen et al. [11], who, using an in situ-loop vascular perfusion of rat small intestine, showed that Na^+-free solutions on the luminal side inhibited GSH transport by about 75%.

On the other hand, Na^+ activation cannot be explained as a stimulation effect on the Na^+-H^+ antiport system, since a GSH proton-driven system was excluded from the results achieved when using differing extravesicular pH (5.5–9). This is in agreement with the results reported for GSH uptake in intestinal tissue and renal BBMV [14,11].

Since GSH at all examined pH values is an anion, we believe that its binding sites on the transport system are mainly electrostatic in nature and that therefore the effect of pH changes could be mediated by changing the ionization state of certain groups at the carrier site and of GSH itself. Furthermore, the lack of GSH binding on the membrane surface in the presence of Na^+ or Ca^{2+} is possibly due to the interactions of the latter with the negative charges of the transporter and of GSH itself. Thus, both the pH 7.5 and the presence of cations may induce an optimal functional conformational state in the cotransporter.

The action mechanism of these cations seems, however, to be rather complex and to differ from each other. The role of Ca^{2+} in terms of an imposed electrical potential difference across BBMV on GSH transport is different from that of monovalent cations. Indeed, experiments performed by valinomycin-induced K^+ diffusion potential or by anion substitution showed that the GSH transport system was an electroneutral process in the presence of Na^+ or K^+, but that it was electrogenic in the presence of Ca^{2+} or in the absence of extravesicular cations.

From these results we can suppose that Na^+ or K^+ are cotransported with GSH and that they thus neutralize the GSH negative charge. It is evident that Ca^{2+} (even supposing it were cotransported) does not neutralize the GSH negative charge. The increase in V_{max} confirms cotransport with the cations [33], even if there is no accumulation of GSH inside the vesicles. However, we cannot exclude the possibility of an exchange system with other cations or anions. These data contrast with what has been reported for Na^+-independent GSH transport systems in renal BBMV and liver canalicular membrane vesicles or for Na^+-dependent GSH transport in renal BLMV, in which GSH transport is electrogenic [14–16]. However, there are no data on the role of membrane potential on intestinal GSH and other electrically charged peptide transporters. Recently, it has been demonstrated that Ca^{2+}, Mn^{2+}, and Mg^{2+} decrease membrane fluidity and that this effect is highly specific for Ca^{2+}, presumably involving the binding of Ca^{2+} to such anionic sites as acidic phospholipid head groups and sialic acid residues in the lipid bilayers of rat small intestine [34]. It has also been reported that changes in fluidity influence a number of enzymic activities and Na^+-dependent glucose transport [31]. Moreover, Ca^{2+} has been demonstrated as not modulating the fluidity of the intestinal basolateral membranes.

Ca^{2+} also influences the fluidity of the controluminal (sinusoidal and contiguous) but not of the luminal (canalicular) plasma membranes of hepatocytes [35]. Some authors report that Ca^{2+} stimulates GSH transport across liver sinusoidal plasma membranes [32]. These results indicate that the effect of Ca^{2+} on fluidity is selective for certain antipodal membranes and suggest that the changes induced by Ca^{2+} on the activity of membrane enzymes and transporters may be pnysiologically important. Ca^{2+} may interact with the lipoproteic microenvironment of the GSH transporter and/or with its hydrophobic surface areas without interact directly with GSH. This may explain the increasing transportability and the electrogenic nature of transport observed in the presence of Ca^{2+}.

The above could therefore indicate that the lipid component of GSH transport systems is important for the transport mechanism, as has been demonstrated for D-glucose Na^+-dependent transport in intestinal BBMV [36]. Moreover, the experiments performed with GSH analogues suggest that GSH transport in intestinal BBMV differs greatly from that in intestinal or renal BLMV [15,11]. It is not inhibited by ophthalmic acid or by probenecid or γ-glutamyl compounds. On the other hand, it is markedly inhibited by S-substituted GSH, as are the GSH transport systems in renal BBMV and in liver canalicular membrane vesicles [14,16]. These observations suggest that this transport system has high affinity with GSH and that the most important structural feature for transport is not the γ-glutamyl group but the tripeptide moiety of GSH and its derivatives.

Previously, we demonstrated that the transport system in question is inhibited neither by the three amino acids (Glu, Cys, Gly) nor by other di- or tri-peptides [10]. The presence of transport systems for GSH S-conjugates has recently been demonstrated in erythrocyte membrane [37], in hepatic canalicular membrane [38] and in renal basolateral membrane [39]. Our results suggest that GSH and its derivatives use the same transport system, thus contributing actively to the intestinal detoxication function of the electrophilic xenobiotics (the small intestine has high activity of GSH-S-transferases [40]). This interesting possibility should be studied further.

We think that GSH monoethyl ester, a competitive inhibitor with an apparent K_i value (33 μM) that is very similar to that of the K_m for GSH (20 μM), is also a substrate of the GSH transport system; this GSH derivative could thus be a therapeutically effective com-

pound after oral administration, as suggested by Meister [41] and Teicher [42]. Indeed Meister demonstrated that this compound is not toxic and that it increases cellular GSH levels, especially in the kidney and liver, by its subsequent hydrolysis into the cells. Unlike GSH, it does not inhibit endogenous GSH synthesis and it enters the liver, in which GSH enacts the main detoxication compound function.

Summarising, GSH transport in intestinal BBMV is Na^+-independent, pH-dependent and specifically activated by mono- and bi-valent cations, in particular by Ca^{2+}; moreover, it is affected by membrane potential in relation to the presence of certain cations and is inhibited in a highly specific way by GSH S-derivatives and by GSH monoethyl ester.

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