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Na^+-DEPENDENT TRANSPORT OF GLYCINE IN RENAL BRUSH BORDER MEMBRANE VESICLES

EVIDENCE FOR A SINGLE SPECIFIC TRANSPORT SYSTEM

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The uptake of glycine in rabbit renal brush border membrane vesicles was shown to consist of glycine transport into an intravesicular space. An Na^+ electrochemical gradient (extravesicular>intravesicular) stimulated the initial rate of glycine uptake and effected a transient accumulation of intravesicular glycine above the steady-state value. This stimulation could not be induced by the imposition of a K^+, Li^+ or choline$^+$ gradient and was enhanced as extravesicular Na^+ was increased from 10 mM to 100 mM. Dissipation of the Na^+ gradient by the ionophore gramicidin D resulted in diminished Na^+-stimulated glycine uptake. Na^+-stimulated uptake of glycine was electrogenic. Substrate-velocity analysis of Na^+-dependent glycine uptake over the range of amino acid concentrations from 25 μM to 10 mM demonstrated a single saturable transport system with apparent $K_m = 996$ μM and $V_{max} = 348$ pmol glycine/mg protein per min. Inhibition observed when the Na^+-dependent uptake of 25 μM glycine was inhibited by 5 mM extravesicular test amino acid segregated dibasic amino acids, which did not inhibit glycine uptake, from all other amino acid groups. The amino acids D-alanine, D-glutamic acid, and D-proline inhibited similarly to their L counterparts. Accelerative exchange of extravesicular [^{3}H]glycine was demonstrated when brush border vesicles were preloaded with glycine, but not when they were preloaded with L-alanine, L-glutamic acid, or with L-proline. It is concluded that a single transport system exists at the level of the rabbit renal brush border membrane that functions to reabsorb glycine independently from other groups of amino acids.

Introduction

Studies of renal tubular transport of glycine and L-proline in preparations of renal cortical slices and isolated renal tubular segments have established the presence of multiple transport systems which mediate the reabsorption of these amino acids, some with specificities for both glycine and imino acids [1–3]. The interpretation of these findings is complicated by the inability of these in vitro techniques to distinguish between amino acid transport across the renal tubular brush border and basal-lateral membranes and by intracellular metabolism of amino acids which may influence transport measurements. To obviate these difficulties, McNamara et al. [4,5] studied Na^+-dependent L-proline and glycine uptakes in isolated rat renal brush border membrane vesicles. Uptakes of L-proline and glycine consisted of

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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transport into vesicles and binding to vesicles. Substrate-velocity analysis of L-proline and glycine uptakes demonstrated the presence of high and low affinity uptake systems for both L-proline and glycine in the isolated membrane vesicles. Inhibition of L-proline uptake by glycine and inhibition of glycine uptake by L-proline were demonstrated by measuring inhibition of Na^+-stimulated L-[^{14}C]proline and [^{14}C]glycine uptake by extravesicular glycine and L-proline.

We have previously studied Na^+-dependent L-proline transport in isolated rabbit renal brush border membrane vesicles [6]. Only minimal binding of L-proline to rabbit membrane vesicles was detected. Substrate velocity analysis of L-proline transport over a range of substrate concentrations which completely overlapped the two ranges used in the study of McNamara et al. described a single transport system for L-proline. Na^+-dependent transport of L-[^{3}H]proline was inhibited more by extravesicular L-proline and hydroxy-L-proline than by extravesicular glycine.

Because of the importance of defining the amino acid specificities of glycine and L-proline transport systems in the renal brush border membrane and because of the differences between our studies of L-proline transport in brush border membrane vesicles and the studies of McNamara et al. we elected to study the transport of glycine in isolated membrane vesicles and define the amino acid specificities of the glycine transport system. We detected no binding of glycine to brush border vesicles. As was the case for L-proline, Na^+-dependent glycine transport in rabbit brush border vesicles occurred by means of a single transport system. This transport system was specific for glycine.

Materials and Methods

Preparation of brush border membrane vesicles. Rabbit renal brush border membrane vesicles were isolated by a sucrose density gradient technique described previously [7,8]. Following each of the last three centrifugations the membranes were suspended in a solution consisting of 300 mM mannitol, 1 mM Hepes-Tris (1 mM Hepes adjusted with Tris hydroxide) or other solutions as indicated in the text. The quality of the preparations was randomly evaluated by specific enzyme markers [9] and occasionally by electron microscopy. Enrichment of purified membrane vesicles compared to renal cortex homogenate was 12–18-fold for trehalase and γ-glutamyltransferase.

Measurement of amino acid transport. Uptake of amino acids was measured by a Millipore filtration technique which has been previously described in detail [6,8]. In the present study, 10 μl of membrane suspension were warmed for 1 min at 20°C and the incubation at 20°C was initiated by the addition of 40 μl of 300 mM mannitol, 1 mM Hepes-Tris pH 7.5 or other solutions as indicated in the text, which contained [^{3}H]glycine and other constituents as indicated. The additions replaced mannitol isosmotically. NaCl, KCl, LiCl or choline chloride gradients (extravesicular > intravesicular) were established by isosmotic substitution of 100 mM salt for mannitol. Incubations were terminated and the Millipore filters washed with 154 mM NaCl/1 mM Hepes-Tris, pH 7.5. Values for nonspecific retention of radioactivity on the filters (less than 0.03% of the total radioactivity in the incubation mixture) were subtracted from the values of the incubated samples. All incubations were carried out in triplicate with freshly prepared brush border membrane vesicles. Each experiment was performed on at least three separate occasions with different membrane preparations.

Protein was assayed by the method of Lowry et al. [10] using bovine serum albumin as the standard.

Initial rates of amino acid uptake measured after 30 s of incubation varied linearly with brush border membrane protein concentrations over the range used in experiments.

Differences between experimental means were analyzed using Dunnett's multiple comparison procedure unless otherwise indicated [11].

Identification and recovery of amino acids transported in brush border vesicles. Following incubation for 30 s and 90 min with [^{3}H]glycine, membrane vesicles were retained on Millipore filters and amino acids were extracted as described elsewhere [12]. The extracts were chromatographed on thin-layer cellulose plates with solvent systems consisting of isopropanol/formic acid/water (40:2:10, v/v). Greater than 90% of the radioactivities taken up by membrane vesicles had R_F

values identical with authentic glycine after both 30 s and 90 min of incubation. No other discreet radioactive spots were detected. These findings indicate that glycine was not metabolized by the membrane vesicles.

Materials. [^{3}H]Glycine (9.39 Ci per mmol) was obtained from New England Nuclear, Boston, MA. Valinomycin and gramicidin-D were obtained from Sigma Chemical Company, St. Louis, MO. Other chemicals were of the highest purity available from commercial sources. All water used for preparing media and reagents was triple distilled and deionized, and all solutions were filtered through 0.45 μM Millipore filters [13] prior to use.

Results

Na^+-gradient dependent uptake of glycine

The uptake of 25 μM [^{3}H]glycine in rabbit renal brush border membrane vesicles was determined as a function of time in the presence of initial 100 mM NaCl and 100 mM KCl gradients (Fig. 1). In the presence of an initial KCl gradient [^{3}H]glycine uptake in membrane vesicles increased over time and a steady state of uptake was reached by 90 min. Uptake of [^{3}H]glycine at early times was enhanced by imposition of an initial NaCl gradient and an 'overshoot' of [^{3}H]glycine uptake was observed [9]. At the peak of the Na^+-stimulated overshoot (2 min) the accumulation of glycine was 1.3 times the value at 90 min. Uptakes of [^{3}H]glycine in the presence of initial NaCl and KCl gradients were equal after 90 min of incubation (steady state).

Na^+-stimulated glycine uptake was a linear function of time for at least 1 min of incubation. The value at 30 s was used to approximate the unidirectional initial rate of glycine uptake.

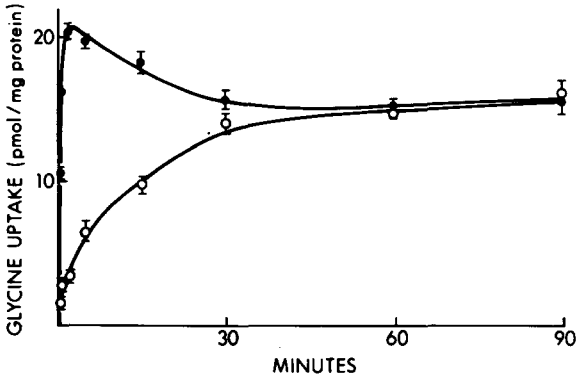

Fig. 1. The time course of 25 μM [^{3}H]glycine uptake in rabbit renal brush border membrane vesicles. Uptake was measured in the presence of an initial 100 mM NaCl (●) or KCl (○) gradient (extravesicular>intravesicular). Data are reported as mean±S.E. of three experiments.

Specificity of the Na^+ stimulation

The specificity of extravesicular Na^+ in inducing an enhancement of [^{3}H]glycine uptake in brush border membrane vesicles is demonstrated in Table I. There was an increased stimulation of initial rates of uptake with increased concentrations of NaCl over the range of 10 mM NaCl to 100 mM NaCl. Substitution of 100 mM KCl, LiCl or choline chloride for NaCl resulted in initial rates of [^{3}H]glycine uptake not significantly different from control (no salt). These results demonstrated a specificity of the glycine transport system for Na^+ in contrast to other monovalent cations and are in agreement with results obtained in studies of other Na^+-dependent amino acid transport systems in brush border vesicles [6,12,14,15].

TABLE I

EFFECTS OF CATIONS ON THE INITIAL RATE OF [^{3}H]GLYCINE UPTAKE

Initial rates of 25 μM [^{3}H]glycine uptake were measured as described in the text. Uptake in the presence of 300 mM mannitol (control) was compared to the uptake observed when the mannitol was replaced isosmotically by each of several Cl^- salts. Data are reported as mean±S.E. of three experiments.

Salt	Concn. (mM)	[^{3}H]Glycine uptake (pmol glycine/mg protein)	
		30 s	90 min
Control	–	3.4±0.24	14.1±1.7
NaCl	100	9.5±0.69 [a]	14.7±0.9
NaCl	50	5.6±0.72 [a]	15.1±1.2
NaCl	10	4.1±0.40 [b]	16.1±2.2
KCl	100	4.0±1.0 [b]	15.1±1.2
LiCl	100	4.7±0.9 [b]	14.9±3.1
Choline Chloride	100	4.2±1.6 [b]	13.8±0.9

[a] Different from control, $P<0.05$.
[b] Different from control, P not significant.

The effect of gramicidin D on Na^+-gradient-dependent glycine uptake

The ionophore gramicidin D, known to increase the cation permeability of biological and artificial membranes [16] was used to enhance membrane conductance for Na^+. When gramicidin D (8 μg per mg membrane protein) was added to an incubation mixture containing an initial 100 mM NaCl gradient, the ionophore decreased the [^{3}H]glycine overshoot (Fig. 2). The presence of gramicidin D had no effect on the steady-state level of [^{3}H]glycine uptake. Gramicidin D presumably dissipated the Na^+ gradient by accelerating the electrogenic entry of Na^+ into membrane vesicles via a pathway not coupled to the uptake of [^{3}H]glycine. The gramicidin D-induced reduction of the [^{3}H]glycine overshoot demonstrated that it was the Na^+ electrochemical gradient that was crucial in stimulating uphill transport of [^{3}H]glycine in membrane vesicles.

Response of glycine uptake in brush border vesicles to conditions of increasing extravesicular osmolality

The effect of extravesicular osmolality on [^{3}H]glycine uptake at steady state (90 min) was determined by incubating brush border membrane vesicles in sucrose solutions of varying osmolalities so as to decrease intravesicular space (Fig. 3) [9]. The uptake of [^{3}H]glycine was inversely proportional to extravesicular osmolality from 300 to 860 mM in the presence and absence of 100 mM NaCl.

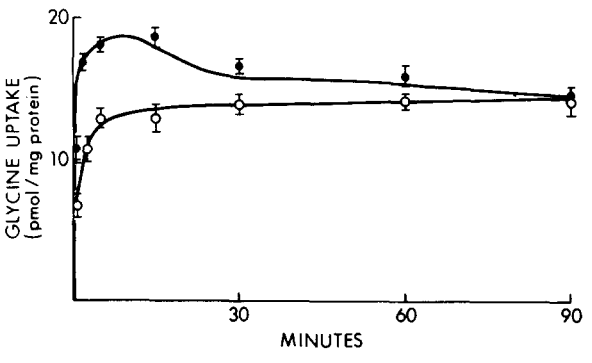

Fig. 2. The effect of gramicidin D on Na^+-gradient-dependent [^{3}H]glycine uptake. Glycine uptake was studied as a function of time in the presence (○) and absence (●) of gramicidin D. Gramicidin D was added to brush border vesicles in 95% ethanol. Ethanol alone was added to control vesicles. Data are reported as mean ± S.E. of three experiments.

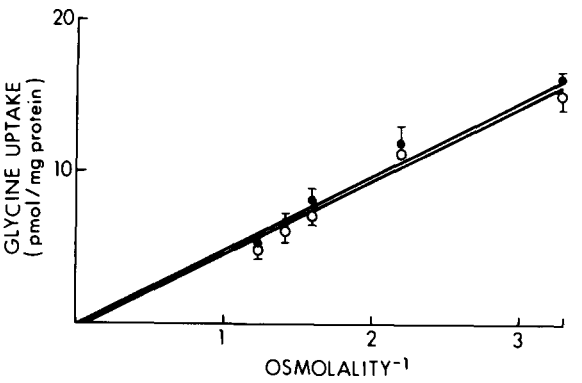

Fig. 3. Steady-state (90 min) uptake of 25 μM [^{3}H]glycine in the presence of an initial 100 mM NaCl gradient (●) and in the presence of mannitol alone (○) plotted as a function of the reciprocal of the extravesicular osmolality. Extravesicular osmolality was adjusted with sucrose. Data are reported as mean ± S.E. of three experiments. Regression lines were calculated by the least-squares method (regression coefficients were 0.995 and 0.988, respectively).

Extrapolation to infinite extravesicular osmolality (zero intravesicular space) estimated no residual [^{3}H]glycine uptake. Thus, uptake of [^{3}H]glycine at steady state could be accounted for by intravesicular accumulation of [^{3}H]glycine.

The effect of valinomycin on glycine uptake

The ionophore valinomycin mediates the electrogenic movement of K^+ down its concentration gradient, thus establishing an electrochemical potential across a membrane [16,17]. Renal brush border vesicles were 'preloaded' with 100 mM K^+ by means of suspension in 50 mM K_2SO_4, 150 mM mannitol, 1 mM Hepes-Tris, pH 7.5 during the final three centrifugations of the preparation procedure [18]. The uptake of [^{3}H]glycine in the preloaded vesicles was measured as a function of time under conditions of an initial 100 mM Na^+ gradient (50 mM Na_2SO_4). The addition of valinomycin (8 μg per mg membrane protein) produced an overshoot with more rapid accumulation of [^{3}H]glycine at early time points (Fig. 4). The steady-state [^{3}H]glycine uptakes in control, and valinomycin-treated vesicles were not different. Valinomycin exerted no effect on Na^+-stimulated [^{3}H]glycine uptake into vesicles which were not preloaded with K^+ (data not shown). These findings suggest that Na^+-stimulated glycine transport

in brush border vesicles is electrogenic; i.e. the co-transport of glycine and Na^+ across the brush border membrane is associated with the net transfer of a positive charge.

Relationship between glycine concentration and initial rates of transport

The effect of different concentrations of glycine on the initial rate of [^{3}H]glycine transport is illustrated in Fig. 5. Initial rates of transport were measured in the presence and absence of an initial 100 mM NaCl gradient. In the absence of NaCl the initial rate of [^{3}H]glycine uptake increased linearly with increasing glycine concentration. In the presence of a NaCl gradient the relationship between [^{3}H]glycine uptake and glycine concentration was non-linear, providing evidence for saturability. When the initial rates of transport observed in the absence of NaCl were subtracted from those measured in the presence of NaCl a curve (Fig. 5, dashed line) was obtained which described a completely saturable Na^+-dependent glycine transport system. When these data were plotted according to Lineweaver-Burk or Eadie-Hofstee (Fig. 5, insets) a single transport system for glycine with an apparent K_m of 996 μM and apparent V_{max}, 348 pmol glycine/mg protein per min was described.

Effect of extravesicular amino acids on the initial rate of glycine uptake

The effect of 5 mM extravesicular amino acids on Na^+-dependent uptake of 25 μM [^{3}H]glycine in brush border vesicles was examined (Table II).

Neutral and acidic amino acids and imino acids inhibited the initial rates of Na^+-dependent glycine uptake. No such inhibition was exerted by either of the dibasic amino acids L-arginine or L-lysine. D-Alanine, D-glutamic acid and D-proline inhibited no differently from their L counterparts.

Glycine accelerative exchange diffusion

Membrane vesicles were 'preloaded' with 1 mM glycine, 1 mM L-alanine, 1 mM L-proline, 1 mM L-glutamic acid or no amino acid by suspending

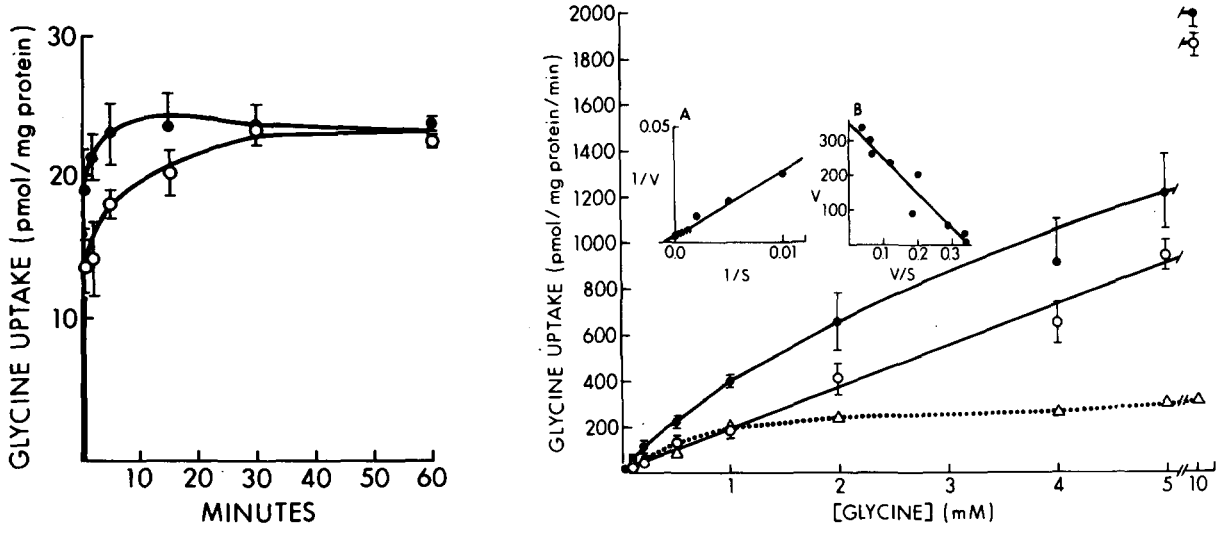

Fig. 4. The effect of valinomycin on Na^+-dependent [^{3}H]glycine uptake. Renal brush border vesicles were preloaded with K^+ as described in the text. The uptake of 25 μM [^{3}H]glycine as a function of time was determined in vesicles to which valinomycin had been added (●) and in the absence of valinomycin (○). Valinomycin was added to vesicles in 95% ethanol. Control vesicles received ethanol alone. Data are expressed as mean ± S.E. for three experiments.

Fig. 5. Glycine concentrations curve. The effect of variation of the concentration of glycine upon initial rates of [^{3}H]glycine uptake was determined in the presence of an initial 100 mM NaCl gradient (●) and in the absence of salt (○). To obtain the middle curve (△) the uptake in the absence of the NaCl gradient was subtracted from that observed in the presence of salt. Data are expressed as mean ± S.E. The insets show data plotted according to (A) Lineweaver-Burk and (B) Eadie-Hofstee. Regression lines were calculated by the least-squares method (regression coefficients were 0.999 and 0.927, respectively).

TABLE II

EFFECT OF EXTRAVESICULAR AMINO ACIDS ON THE INITIAL RATE OF GLYCINE UPTAKE

The initial rate of 25 μM [^{3}H]glycine uptake was measured in the presence and absence of 5 mM test amino acid under conditions of initial 100 mM NaCl gradients (extravesicular> intravesicular) and in the absence of salt. Initial rates of uptake measured in the absence of salt gradients were subtracted from those measured in the presence of NaCl gradients to obtain the Na^+-dependent uptake. The results are expressed as % Na^+-dependent uptake measured in the absence of test amino acid (control).

Test amino acid	Concn. (mM)	Na^+-dependent [^{3}H]glycine uptake (% of control)
None	–	100
Glycine	5	41 ± 4.2
L-Alanine	5	27 ± 8.1
D-Alanine	5	39 ± 7.2 [a]
L-Proline	5	40 ± 12.6
D-Proline	5	56 ± 3.7 [a]
L-Glutamic acid	5	73 ± 2.2
D-Glutamic acid	5	76 ± 7.6 [a]
L-Arginine	5	96 ± 4.9
L-Lysine	5	99 ± 8.7

[a] No difference between L- and D-amino acid (Student's t-test).

vesicles in solutions containing 300 mM mannitol, 1 mM Hepes-Tris, pH 7.5, 1 mM amino acid or no amino acid, during the final three centrifugations of the preparation procedure [18]. The uptake of [^{3}H]glycine was measured at 30 s (initial rate) in each case. There was a marked stimulation of the initial rate of [^{3}H]glycine uptake over that observed in the absence of a preload when membrane vesicles were preloaded with glycine but not with other amino acids. Thus, accelerative exchange of extravesicular [^{3}H]glycine was demonstrated for intravesicular glycine but not for other amino acids (Table III).

Discussion

In the present studies substrate-velocity analysis of Na^+-dependent glycine transport in rabbit renal brush border membrane vesicles defined a single transport system for glycine. We cannot explain the difference between this finding and the findings of McNamara et al. [4,5] who described two glycine transport systems in rat brush border vesicles, except to implicate binding of glycine to membrane vesicles as a factor which might have complicated the interpretation of data in the latter

TABLE III

EFFECT OF PRELOADING WITH UNLABELLED AMINO ACID ON THE UPTAKE OF [^{3}H]GLYCINE

Renal brush border membrane vesicles were 'preloaded' with 100 mM NaCl, 100 mM mannitol, 1 mM Hepes-Tris, pH 7.5, containing 1 mM glycine, 1 mM L-alanine, 1 mM L-proline, 1 mM L-glutamic acid, or no amino acid. Then 40 μl volumes of [^{3}H]glycine, 100 mM mannitol, 1 mM Hepes-Tris, pH 7.5 were added to 10 μl volumes of the membrane vesicles such that the initial concentration of extravesicular [^{3}H]glycine was 5 μM and extravesicular amino acid was 200 μM. Uptake of 5 μM [^{3}H]glycine was determined at 30 s and 90 min. The [^{3}H]glycine uptake measured when preincubation was with no amino acid was considered the control value (100%). Data are reported as mean ± S.E. of three experiments.

Preloading amino acid (concentration)	Extravesicular amino acid (μM)	Uptake (% of control)	
		30 s	90 min
None	Glycine (200)	100	100
Glycine (1 mM)	Glycine (200)	142 ± 3.2 [a]	100 ± 4.1
None	L-Alanine (200)	100	100
L-Alanine (1 mM)	L-Alanine (200)	93 ± 7.0	114 ± 3.7
None	L-Proline (200)	100	100
L-Proline (1 mM)	L-Proline (200)	96 ± 3.6	100 ± 1.8
None	L-Glutamic acid (200)	100	100
L-Glutamic acid (1 mM)	L-Glutamic acid (200)	93 ± 2.1	105 ± 2.7

[a] Different from control, $P<0.05$.

studies or to implicate species differences between the rabbit and rat.

Although the Na^+-dependent transport of extravesicular [^{3}H]glycine was inhibited by several D- and L-extravesicular amino acids, accelerative exchange diffusion of extravesicular [^{3}H]glycine was demonstrated for intravesicular glycine only. It has been shown in several membrane systems including brush border vesicles that substances which share a common carrier mechanism stimulate accelerative exchange of one for another across the membrane [18–20]. Our data are consistent with the presence of an Na^+-dependent glycine transport system in the rabbit renal brush border membrane which is specific for glycine.

The inhibition of initial rates of [^{3}H]glycine transport in brush border vesicles by extravesicular amino acids could result from several mechanisms. Co-transport of Na^+ with L-alanine or L-proline would be expected to dissipate the electrochemical Na^+ gradient and in doing so inhibit the Na^+-dependent electrogenic transport of glycine [21]. We have previously demonstrated that Na^+-L-glutamic acid co-transport in rabbit brush border vesicles was electroneutral [15], thus in our brush border membrane transport system, Na^+-L-glutamic acid transport would not be expected to dissipate the Na^+ electrical gradient. However, the co-transport of L-glutamic acid with Na^+ would be expected to dissipate the chemical Na^+ gradient and in this manner inhibit the initial rate of glycine transport [15,21].

We have previously demonstrated Na^+-dependent transport of D-alanine in rabbit renal brush border vesicles [14]. The Na^+-dependent transport of D-alanine was not as rapid as the Na^+-dependent transport of L-alanine [14]. For this reason, extravesicular D-alanine and perhaps other D-amino acids would not be expected to dissipate the electrochemical Na^+ gradient as rapidly as L-amino acids and therefore should not inhibit initial rates of Na^+-dependent glycine transport as much as their L-counterparts if inhibition were mediated solely through dissipation of the Na^+-gradient. Our observations to the contrary in the present studies suggest that extravesicular D- and L-amino acids inhibit Na^+-dependent [^{3}H]glycine transport by a mechanism in addition to dissipation of the Na^+ gradient. It is unlikely that D-amino acids share the Na^+-dependent glycine transport system since (a) D-amino acids are not significantly reabsorbed by the mammalian nephron [22] and (b) several L-amino acids were shown not to share the Na^+-dependent glycine transport system with glycine in the present study. It is possible, however, that both D- and L-amino acids interfere with the binding of glycine to a putative membrane carrier without themselves being transported by that carrier. Since glycine has no asymmetric carbon atom it would be equally subject to such inhibition by D- and L-amino acids.

Because of the multiple possible mechanisms of extravesicular amino acid inhibition of [^{3}H]glycine transport in brush border vesicles, the results of studies which measure inhibition in this manner must be interpreted with caution. Thus, the data of McNamara et al, which were generated in this way may be subject to alternative explanation. These investigators measured the effect of extravesicular L-proline and glycine on the Na^+-stimulated uptake of [^{14}C]glycine and L-[^{14}C]proline in brush border membrane vesicles. They concluded on the basis of substrate-velocity analysis of ^{14}C-labeled amino acid uptake in the presence and absence of competing amino acid, that L-proline and glycine shared one of two glycine transport systems in the brush border membrane. For the reasons cited above, this conclusion may be in error.

Familial hyperglycinuria is a rare disorder of renal amino acid reabsorption characterized by glycinuria in the absence of iminoaciduria [23,34]. It has been previously characterized as a disorder of the imino-glycine transport system [24], but the data presented in these studies support the contention that it may represent an abnormality of an unshared glycine transport mechanism [5].

Acknowledgements

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