Biochimica et Biophysica Acta 814 (1985) 186–190
Elsevier

BBA 70212

BBA Report

Proton-coupled transport of glycylglycine in rabbit renal brush-border membrane vesicles

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(Received November 13th, 1984)

Key words: Peptide transport; Glycylglycine; H^+-cotransport; Brush-border membrane; (Rabbit kidney)

Transport of glycylglycine into rabbit renal brush-border membrane vesicles was found to be Na^+-independent, H^+ gradient-dependent and electrogenic. Marked overshoot uptake of the dipeptide was observed when an inward-directed proton gradient and inside-negative potential difference were imposed simultaneously across the vesicular membranes. Saturable depolarization of vesicular membranes could be demonstrated with glycylglycine by use of a fluorescent cyanine dye, di-S-C_3(5). The results indicate that glycylglycine is contransported with H^+ across the membranes.

Carrier-mediated transport of intact di- or tripeptides is well established in mammalian small intestinal [1,2] and renal [3,4] brush-border membranes. Previous investigations revealed that most of dipeptides so far examined were transported by a Na^+-independent mechanism [3–9]. Recently, Ganapathy et al. demonstrated that transport of glycyl-L-proline, L-carnosine [10] and glycylsarcosine [11] by intestinal or renal brush-border membrane vesicles was stimulated by an inward-directed H^+ gradient, suggesting the presence of H^+-coupled cotransport mechanism for these dipeptides. However, in their studies, uphill transport of dipeptides could not be demonstrated even in the presence of a gradient of 2 pH units. In the present study, we found that transport of another dipeptide, glycylglycine, was also stimulated by a H^+-gradient, and that marked overshoot uptake was demonstrable for this peptide when an inward-directed H^+-gradient and inside-negative K^+ diffusion potential were imposed simultaneously across the membranes. The findings seem to corroborate that glycine-dipeptides are cotransported with H^+ across, at least, renal brush-border membranes.

Brush-border membrane vesicles were prepared from rabbit renal cortex by a slight modification of the Mg/EGTA-method described by Biber et al. [12]. In brief, 20 g of frozen renal cortex obtained from thoroughly perfused kidneys was homogenized in 100 ml of a solution containing 300 mM mannitol, 5 mM EGTA and 12 mM Tris-HCl (pH 7.5), by use of a polytron-type homogenizer (Physcotron; Nition Co. Ltd., Japan). The homogenate was then mixed with 120 ml of ice-cold deionized distilled water, to which $MgCl_2$ was added to the final concentration of 10 mM. After standing on ice for 15 min, the sample was centrifuged at $4000 \times g$ for 10 min. The super-

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Abbreviations: di-S-C_3(5), 3,3'-dipropylthiodicarbocyanine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

natant was then centrifuged at 30000 × *g* for 30 min. The pellet was washed twice in a buffer containing 100 mM K_2SO_4, 0.05 mM $MgSO_4$ and 50 mM Hepes/Tris (pH 7.5), and finally resuspended in the same buffer at the protein concentration of about 10 mg/ml and stored in liquid nitrogen until use. The enrichment of specific activities (final pellet/homogenate) of the brush-border marker enzymes, alkaline phosphatase and trehalase, was 10–13-fold, while that of the basolateral membrane marker, $(Na^+ + K^+)$-ATPase was consistently below 1.0. Protein concentrations were measured according to the method of Bradford [13] with bovine serum albumin as a standard. Alkaline phosphatase was assayed by the method of Bessey et al. [14], trehalase by the method of Dahlqvist [15] and $(Na^+ + K^+)$-ATPase by the method of Quigley and Gotterer [16]. Uptake of glycylglycine (Gly-Gly) by the membrane vesicles was measured with [1-^{14}C]Gly-Gly at 25°C by the rapid filtration method as originally described by Hopfer et al. [17] using 0.45 μm pore-sized millipore filters. Specific conditions of incubation for each experiment are given in the legends to figures. Changes in transmembrane potential of the membrane vesicles were measured by using a membrane potential-sensitive cyanine dye, di-S-C_3(5), according to the method described by Beck and Sacktor [18], and a Hitachi fluorospectrophotometer (Type 650-105). The excitation wave length was set at 622 nm and the emission was measured at 670 nm. Valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma and Aldrish, respectively. [1-^{14}C]Gly-Gly (custom made) was obtained from Amersham International. Di-S-C_3(5) was supplied from Japan Photosensitive Dye Institute (Okayama, Japan). All other chemicals were of reagent grade purity.

At first, uptake of Gly-Gly was compared in the presence and absence of a Na^+-gradient at different extravesicular pH (pH_o) values. The vesicles used were prepared at pH 7.5 and equilibrated with the same pH before uptake experiments. The uptake of the dipeptide was entirely independent of the presence of a Na^+-gradient at normal pH_o (7.4), confirming our previous finding [9]. Also, no statistically significant difference was seen at pH_o 6.0 between uptake values in the presence and absence of a Na^+ gradient, i.e. the values were 14.3 ± 0.5 pmol/30 s per mg protein in the presence of 100 mM NaSCN and 15.7 ± 0.8 pmol/30 s per mg protein in the presence of 100 mM KSCN (mean ± S.E., $n = 4$). Lowering the pH_o from 7.4 to 6.5 or 6.0 had a marked stimulating effect on the uptake while elevating the pH_o to 8.4 exerted a reverse effect as shown in Fig. 1. The maximum stimulation was seen at pH_o 6.0. The stimulating effect of low pH_o was strongly depressed when a protonophore, FCCP, was added to the incubation media (Table I), indicating that it is the inward-directed H^+-gradient which stimulates Gly-Gly uptake, but not low outside pH per se. This was further ascertained by a separate series of experiments in which glycylglycine uptake was compared at different pH values where no pH gradient existed between the inside and the outside of the vesicles. The data are summarized in Table II. The uptake value at pH 6.0 was not significantly different from that obtained at pH 7.5, confirming the above conclusion.

The uptake was significantly enhanced by valinomycin-induced inside-negative K^+ diffusion potential, either at pH_o 7.5 or 6.0 (Table I), indi-

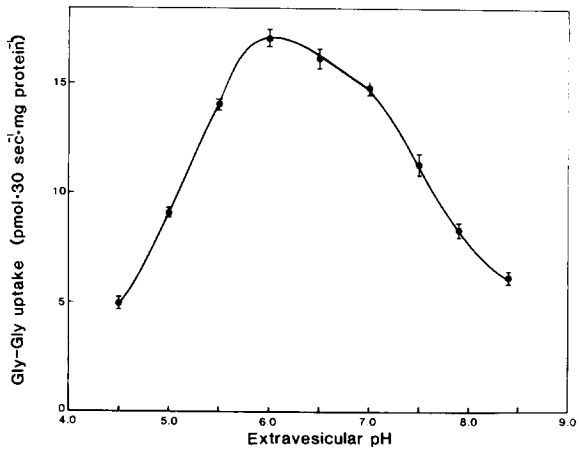

Fig. 1. Effect of extravesicular pH on Gly-Gly uptake by renal brush-border membrane vesicles. The vesicles were preloaded with a 50 mM Hepes/Tris buffer, pH 7.5, containing 100 mM K_2SO_4 and 0.05 mM $MgSO_4$. Transport was assayed by incubating vesicles in 50 mM Mes/Hepes/Tris buffers in the range of pH 4.5–6.5, or 50 mM Heper/Tris buffers in the range of pH 7.0–8.4, each containing 45 mM mannitol, 90 mM KSCN and 8 μM [1-^{14}C]Gly-Gly. The incubation time was 30 s.

TABLE I

EFFECTS OF FCCP AND VALINOMYCIN ON Gly-Gly UPTAKE

The membrane vesicles were preloaded with a 50 mM Hepes/Tris buffer (pH 7.5) containing 100 mM K_2SO_4 and 0.05 mM $MgSO_4$. The uptake solutions were composed of either 50 mM Mes/Hepes/Tris buffer (pH 6.0) or 50 mM Hepes/Tris buffer (pH 7.5), 270 mM mannitol, 8 μM [1-^{14}C]Gly-Gly and either 10 μM FCCP, 10 μM valinomycin or 0.5% ethanol (control). Data are expressed as means ± S.E. of four determinations.

	Gly-Gly uptake (pmol/30 s per mg protein)	
	$pH_o = 6.0$	$pH_o = 7.5$
Control	20.4 ± 0.2	8.8 ± 0.6
FCCP	12.8 ± 0.9 ($p < 0.001$)	7.6 ± 0.5 (n.s.[a])
Valinomycin	27.7 ± 1.3 ($p < 0.002$)	11.6 ± 0.8 ($p < 0.05$)

[a] n.s., statistically not different.

cating that the transport is electrogenic. By using a membrane potential-sensitive cyanine dye, di-S-$C_3(5)$, the electrogenic transport was confirmed. Fig. 2 shows an example of recordings of fluorescence changes induced by addition of Gly-Gly to a vesicle suspension containing 2 μM of di-S-$C_3(5)$. A rapid increase in fluorescence was induced by Gly-Gly, the change being indicative of membrane

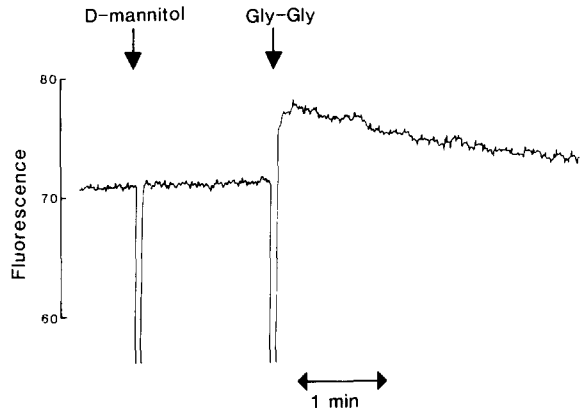

Fig. 2. Effect of Gly-Gly on transmembrane potential as monitored by the fluorescence change of a membrane potential sensitive dye, di-S-$C_3(5)$. The membrane vesicles (protein amount 40.5 μg/ml) were suspended in a solution containing 100 mM mannitol, 100 mM choline chloride, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Hepes/Tris (pH 7.5) and 2 μM di-S-$C_3(5)$. The interruption of the fluorometric traces indicate the addition of either 50 mM mannitol or 43 mM Gly-Gly. A change of 10 arbitrary units represents 14% change in the total fluorescence.

depolarization as confirmed by the valinomycin-induced K^+ diffusion potential. The depolarizing effect of Gly-Gly was unchanged when extravesicular Na^+ was replaced with K^+, choline$^+$, Tris$^+$ or Li^+ (data not shown). L-Glucose or D-mannitol did not induce any change in the fluorescence under the same incubation condition, while D-glucose induced membrane depolarization only when Na^+ was present in the extravesicular solution. These findings indicate that Gly-Gly transport is independent of Na^+ but dependent on the presence of a H^+ gradient and associated with the transfer of net positive charge.

When an inward-directed proton gradient and inside-negative K^+ diffusion potential were imposed simultaneously across the vesicular membranes, marked overshoot uptake of Gly-Gly was observed (Fig. 3). The peak uptake occurred at about 2 min after the start of incubation and the equilibrium was achieved after about 30 min. Such a time course is not much different from that found for Na^+-coupled cotransport of D-glucose [19] or glycine [9]. In the absence of both the pH gradient and the inside-negative membrane potential, the uptake curve was simple without showing overshoot.

TABLE II

EFFECT OF A LOW EXTERNAL pH ON GLYCYLGLYCINE UPTAKE IN THE ABSENCE AND PRESENCE OF A pH GRADIENT ACROSS THE VESICULAR MEMBRANES

The membrane vesicles were preload with a solution containing 300 mM mannitol, 20 mM Hepes/Tris (pH 7.5) or 20 mM Mes/Hepes/Tris (pH 6.0) for 3 h at 20°C and subsequently for 3 h at 4°C. The uptake medium contained 100 mM mannitol, 100 mM KSCN, 20 mM Hepes/Tris (pH 7.5) or Mes/Hepes/Tris (pH 6.0), 0.1 mM Gly-Gly and 2.5 μCi/ml [1-^{14}C]Gly-Gly. The uptake was measured by adding 20 μl of the vesicular suspension to 95 μl uptake medium and incubating the vesicles for 30 s at 25°C. Each datum represents mean ± S.E.

External pH	Internal pH	Gly-Gly uptake (pmol/30 s per mg protein)
7.5	7.5	93.0 ± 6.9
6.0	6.0	91.3 ± 4.9
6.0	7.5	124.8 ± 4.7

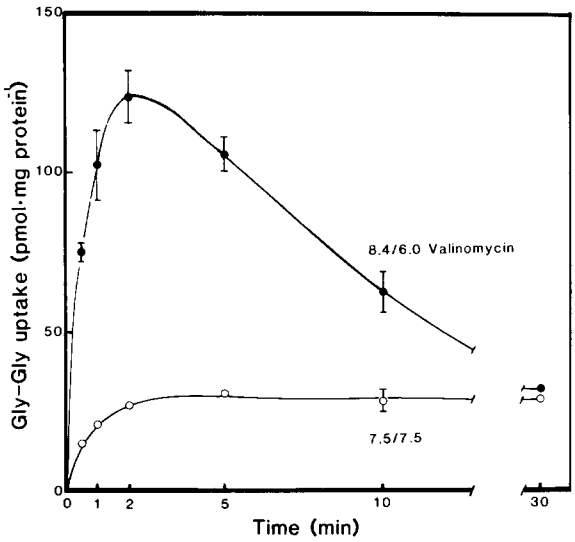

Fig. 3. Time-course of Gly-Gly uptake from a 8 μM Gly-Gly solution in the presence and absence of a pH gradient plus valinomycin-induced K^+ diffusion potential (inside the vesicle negative). The vesicles were preloaded with a 50 mM Hepes/Tris buffer, pH 8.4 (●———●) or pH 7.5 (○———○), containing 100 mM K_2SO_4 and 0.05 mM $MgSO_4$. Uptake was assayed either in a 50 mM Mes/Hepes/Tris buffer (pH 6.0) containing 270 mM mannitol and 10 μM valinomycin (●———●) or in a 50 mM Hepes/Tris buffer, pH 7.5, containing 270 mM mannitol and 0.5% ethanol as control (○———○).

The results of the present study clearly demonstrate that Gly-Gly transport across rabbit renal brush border membrane is Na^+-independent but H^+ gradient-dependent. All of our findings are compatible with the predicted mechanism of cotransport of Gly-Gly with H^+. The most important finding in the report is the overshoot uptake of the substrate which has not been reported thus far. In the present study, we used the Mg/EGTA-method [12] for the preparation of membrane vesicles. As reported by Sabolić and Burckhardt [20], the vesicles prepared by the Ca^{2+}-precipitation methods [21] is more permeable to cations as compared with those prepared by Mg/EGTA-method. Biber et al. [12] also reported higher rates of Na^+-dependent D-glucose or phosphate transport in vesicles prepared by the Mg/EGTA-method than in vesicles prepared by the Ca^{2+}-precipitation method. Our preliminary measurements also revealed that Gly-Gly uptake compared under the same incubation conditions was significantly higher in vesicles prepared by the Mg/EGTA-method than in vesicles prepared by the Ca^{2+} method. Part of reasons of the successful demonstration of H^+-dependent and membrane potential-dependent overshoot may be the use of more tight membrane vesicles. H^+-dependent transport of Gly-Gly was found to be electrogenic as evidenced by enhancement of uptake by valinomycin-induced K^+ diffusion potential and Gly-Gly-induced depolarizations of the vesicular membranes. Recently, Ganapathy et al. [11] also demonstrated glycylsarcosine-induced membrane depolarization in intestinal brush-border membrane vesicles. These findings together with findings of Na^+-independent, H^+-dependent transport for other dipeptides seem to indicate that the H^+-coupled cotransport is a common mechanism of dipeptide transport in both small intestine and kidney.

The renal proximal tubule is known to have capacity to hydrolyze filtered peptides, particularly relatively short linear peptides such as angiotensin I, II and bradykinin, and to absorb the degraded products [22]. This function may be biologically important to conserve amino acids, regulate circulating levels of peptide hormones and inactivate toxic peptides, if present. The transport system for dipeptides may be functioning, at least in part, in the process of absorption of the degradation products. The renal brush-border membrane is also known to have a Na^+/H^+ antiport system [23]. The parallel existence of the Na^+/H^+ antiport and the H^+/dipeptide cotransport systems in the brush-border membrane suggests the possibility that the proximal tubular cells are capable of absorbing dipeptides in a uphill manner depending on the activity of the $(Na^+ + K^+)$-ATPase indirectly.

We wish to thank Dr. M. Himukai for useful discussions. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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