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In vitro peptidase activity of rat mucosa cell fractions against glutamine-containing dipeptides

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Instability and limited solubility hampers the inclusion of free glutamine in aqueous preparations intended for clinical nutrition. This drawback can be overcome by using synthetic stable and highly soluble glutamine-containing dipeptides. Rapid utilization in the target tissue is a prerequisite for their rational use. Hydrolytic capacity of rat small intestine against L-alanyl-L-glutamine and glycyl-L-glutamine was assessed by incubating mucosa cell fractions (brush border and basolateral membrane vesicles, soluble fraction) under controlled and standardized conditions. Subsequently dipeptide disappearance and the simultaneous liberation of the constituent free amino acids were monitored (liquid chromatography) enabling estimation of the Michaelis constants and the maximum velocity of the reaction.

The estimated Michaelis constants (Eadie-Hofstee plot) for membrane-bound peptidases appeared to be lower for alanyl-glutamine than those for glycyl-glutamine (brush border membrane: 0.99 ± 0.23 vs. 4.59 ± 2.01 mmol/L; basolateral membrane: 1.02 ± 0.38 vs. 4.80 ± 0.89 mmol/L). The estimated capacity for hydrolysis, expressed per mg mixed protein, was 3- to 5-fold higher for alanyl-glutamine than for glycyl-glutamine in all preparations.

The results indicate that mammalian intestinal mucosa is well equipped to enable an efficient hydrolysis of glutamine-containing dipeptides either delivered luminally or intravenously. (J. Nutr. Biochem. 7:135-141, 1996.)

Keywords: peptidase; glutamine; dipeptide; basolateral membrane; mucosa

Introduction

In classic work, Windmueller and Spaeth demonstrated that epithelial cells of the small intestine prefer glutamine as a fuel for oxidative metabolism.[1] Later, it was shown that long-term glutamine deficiency is accompanied by mucosal atrophy.[2] Normally, the availability of glutamine may prompt enterocytes to enter the S phase of cell cycle and thus act as a trophic-proliferative factor.[3,4] These facts, together with clinical experience, have resulted in glutamine now being considered as a conditionally essential amino acid, especially during episodes of critical illness or malnutrition.[5] Glutamine nutrition in the context of clinical nutrition is therefore conditionally essential. Currently, addition of free glutamine in its native form to nutritional regimens is hampered because of its instability during commonly used heat sterilization procedures and subsequent long-term storage.[6,7] The use of specifically prepared low concentrated glutamine preparations (cold filtration techniques under strict aseptic conditions) may be problematic in fluid-restricted patients.[6,7] These problems can be overcome by the use of heat stable and highly soluble glutamine-containing dipeptides, glycyl-L-glutamine (Gly-Gln) or L-alanyl-L-glutamine (Ala-Gln),[8-10] which supports the proposition that the provision of peptides of those amino acids (also difficult to deliver in their native form) may be of general importance.

It has been demonstrated that glutamine dipeptides administered by bolus injection are rapidly cleared from plasma.[11-16] Prompt liberation of equimolar amounts of constituent amino acids, as well as inconsequental urinary

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Received January 11, 1995; accepted October 5, 1995.

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0955-2863/96/$15.00
SSDI 0955-2863(95)00195-6

losses of intact dipeptides suggest a virtually complete hydrolysis presumably catalyzed by free[14,17,18] or membrane-bound peptidases probably in liver, kidney,[19] and muscle.* Little is known about the enzymatic equipment concerning hydrolases of blood-facing membranes of epithelial tissues.

The aim of the present study was to assess in vitro the hydrolytic capacity in different mucosal cell fractions of the rat small intestine. Peptidase activities against two glutamine-containing dipeptides, Ala-Gln and Gly-Gln, were investigated in the basolateral membrane, the brush border membrane, as well as the soluble fraction enabling a direct comparison of hydrolase capacities.

Methods and materials

Animals

Male Sprague-Dawley rats (250–350 g, Süddt. Versuchstierfarm, Interfauna, Tuttlingen, Germany) were food-deprived for 12 hr. After decapitation the small intestine was removed, flushed repeatedly with icecold saline, and kept at 4°C. For the preparation of the mucosa, the intestine was opened longitudinally and blotted with absorbent paper to remove mucus and excess fluid. The mucosa was scraped off with a glass slide, frozen in liquid nitrogen, and kept at −70°C until vesicle preparation (not more than 30 days).[20]

Brush border membrane vesicles (BBMV)

BBMV were isolated using a divalent cation precipitation method.[20,21] Briefly, the scrapings (3–5 g) were homogenized in 30 mL phosphate buffer (20 mmol/L K_2HPO_4, 5 mmol/L EGTA, 140 mmol/L NaCl, pH 7.4) with a Potter homogenizer for 5 min and diluted with 120 mL phosphate buffer (crude homogenate). For aggregation of the internal membranes of mitochondria, nuclei, endoplasmatic reticulum, and basolateral membranes, 3 mL of 1 mol/L $MgCl_2$ were added to give a final concentration of 20 mmol/L. After 15 min at 4°C the crude homogenate was centrifuged at 3,000 g for 15 min. The supernatant was spun again at 27,000 g for 30 min and the pellet was resuspended in 30 mL of the phosphate buffer with 10 strokes of a homogenizer. After addition of 0.3 mL 1 mol/L $MgCl_2$ to the suspension, the two centrifugation steps described above were repeated. The pellet was suspended in 30 mL buffer (20 mmol/L K_2HPO_4, 0.9% NaCl, pH 7.4 = incubation buffer) and the brush border membranes were spun down at 48,000 g for 30 min. They were washed once and the final pellet was resuspended in an incubation buffer with a 27-gauge needle and kept at −70°C until biochemical analyses.

Soluble fraction (SF)

An aliquot of the crude homogenate was kept at 4°C for 20 min for enzyme solubilization and the SF that still might contain some microsomes was obtained by centrifugation (27,000 g, 30 min).[22]

Basolateral membrane vesicles (BLMV)

BLMV were isolated according to Wilson and Webb.[23] Mucosa (10–14 g) was homogenized in bicarbonate buffer (10 mmol/L $NaHCO_3$, 250 mmol/L sucrose, 5 mmol/L $MgCl_2$, pH 7.4) with an Ultra-Turrax for 15 s. The homogenate was allowed to stand at 4°C for 30 min with gentle stirring for aggregation of the internal and basolateral membranes that were centrifuged afterwards at 8,700 g (12 min). The pellet was homogenized with 12 strokes of a Potter homogenizer in a buffer containing 10 mmol/L $NaHCO_3$, 250 mmol/L sucrose, 2 mmol/L $MgCl_2$, pH 7.4. The suspension was centrifuged at 750 g for 15 min resulting in a pellet of the internal membranes that are of greater density than the basolateral membranes. The latter were spun down from the supernatant at 8,700 g for 12 min. The crude basolateral membrane fraction was resuspended in 10 mL buffer and applied to a sucrose density gradient (27%, 31%; wt/wt). After centrifugation in a swinging-bucket rotor (SW 27, Beckman) at 105,000 g for 90 min, the membranes were collected at the interface between the buffer and the 27% sucrose layer. The washed membranes were resuspended in incubation buffer and kept at −70°C until enzymatic tests and incubations.

Marker enzymes and protein

Leucine-aminopeptidase (LAP) was chosen as the marker for the brush border membranes.[20,24] LAP activity was determined with an enzyme assay kit (Merck, Darmstadt, Germany) and expressed in kat/g protein.

The marker for the basolateral membranes was the ouabain-sensitive Na^+/K^+-ATPase. The assay described by Colas et al.[25] was employed except that the reaction was stopped with 1 mol/L NaOH.

Protein was estimated using the Bradford method with bovine serum albumin as standard protein.[26]

Incubations

BBMV, SF, and BLMV (30–200 μg protein/mL each) were incubated (60 min) with the dipeptides Ala-Gln and Gly-Gln, respectively (0.25–10 mmol/L; in duplicate) in phosphate buffer (20 mmol/L KH_2PO_4, 140 mmol/L NaCl, pH 7.4) at 37°C. After various time intervals (max. 60 min), aliquots of the incubation mixture (250 μL) were obtained and the reaction was stopped by adding 30% sulphosalicylic acid containing 1 mmol/L norvaline as internal standard. After centrifugation, dipeptide and amino acid concentrations in the supernatants were analyzed by reversed-phase HPLC after precolumn derivatisation with orthophthaldialdehyde/3-mercaptopropionic acid.[27]

Calculations

Initial velocity (v_0) of the hydrolysis was estimated by regression analysis within the linear range of the reaction (1–4 min) and was expressed in nmol/(mg protein * min). Maximum velocity (V_{max}) and the Michaelis constant K_m (mmol/L) in this system were calculated by Eadie-Hofstee method.[28] Mean values ± SD are given.

Results

Characterization of tissue preparation

The specific activity of the marker enzyme LAP was (1.2 ± 0.2) * 10^{-7} kat/g protein in the homogenate, (0.7 ± 0.3) * 10^{-7} kat/g protein in the SF and (18.2 ± 11.3) * 10^{-7} kat/g protein in the BBMV. As expected, the activity of LAP in the SF was very low. The specific activity of the ouabain-sensitive Na^+/K^+ phosphatase in BLMV was (0.8 ± 0.2) * 10^{-7} kat/g protein in the homogenate and (12.0 ± 2.6) * 10^{-7} kat/g protein in the BLMV. The mean enrichment of LAP in BBMV and ouabain-sensitive Na^+/K^+ phosphatase in BLMV was 15 ± 6.2 and 14 ± 1.9-fold, respectively (n =

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4). BLMV also showed little activity of LAP (mean enrichment of 1.2 compared to the homogenate). The combined results indicate satisfactory fractionation of membrane material suggesting no cross contamination.

In vitro dipeptide hydrolysis

Time courses of peptide/amino acid concentrations in the incubation mixtures are given in *Figures 1* and *2*. BBMV, SF, and BLMV exhibited measurable peptidase activities against both Ala-Gln and Gly-Gln. Dipeptide disappearance was accompanied by a virtually equimolar increase of the constituent-free amino acids. Hydrolysis of Ala-Gln was more rapid in all investigated cell fractions.

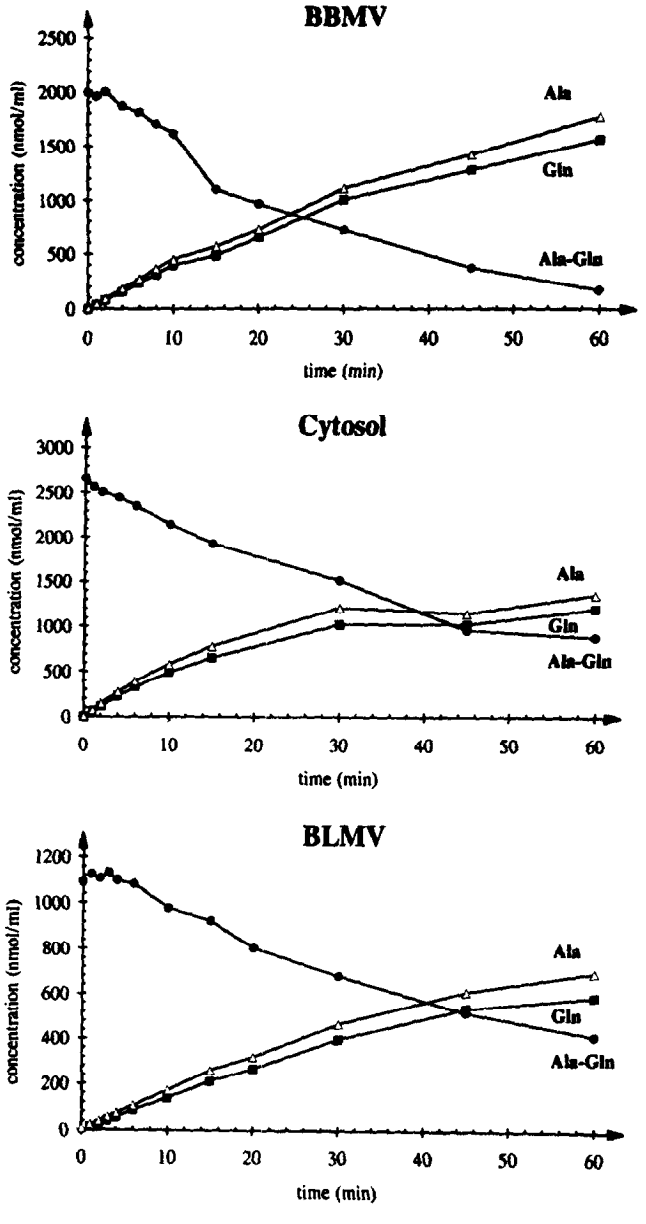

Figure 1 Time course of incubations with Ala-Gln. The concentration of the dipeptide Ala-Gln (●) and liberated amino acids alanine (△) and glutamine (■) was measured at various time points during the incubation. The figure depicts the results of one incubation. The protein concentration in the reaction medium was 83.3 µg prot./mL in BBMV, 73.1 µg prot./mL in the SF, and 97.5 µg prot./mL in BLMV.

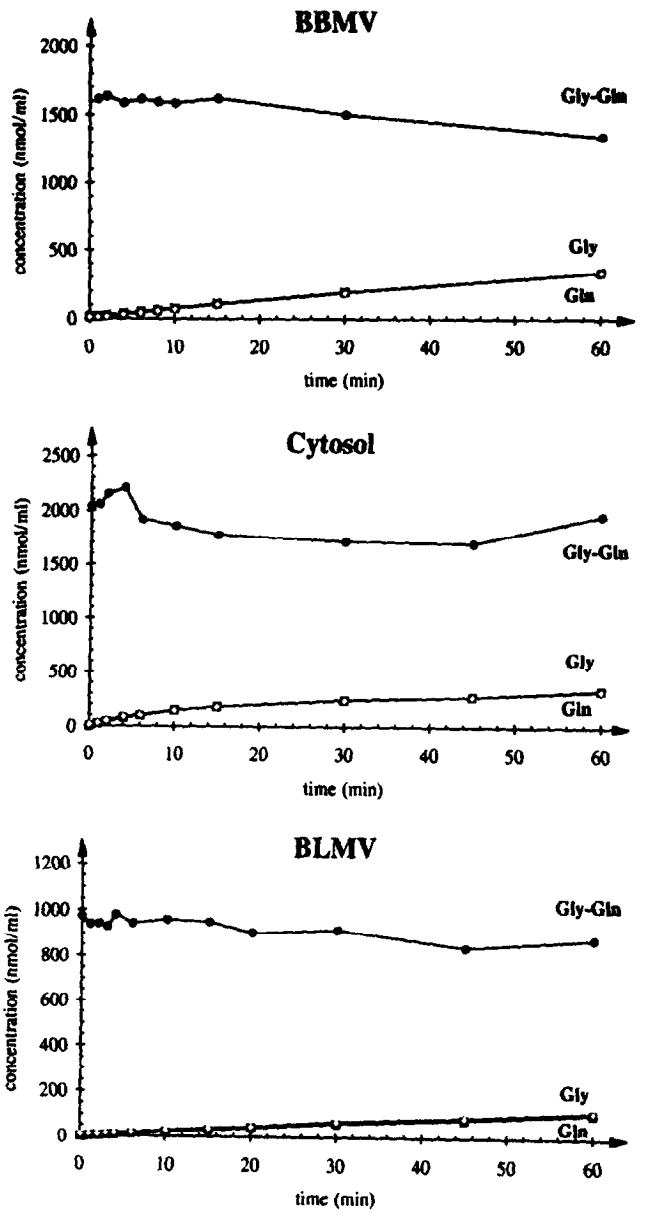

Figure 2 Time course of incubations with Gly-Gln. The concentration of the dipeptide Gly-Gln (●) and liberated amino acids glycine (◇) and glutamine (■) was measured at various time points during the incubation. The figure depicts the results of one incubation. The protein concentration in the reaction medium was 91.9 µg prot./mL in BBMV, 146.1 µg prot./mL in the SF, and 97.5 µg prot./mL in BLMV.

The initial peptidase velocities of BBMV, SF, and BLMV were linearly related to the protein concentration in the reaction medium (*Figure 3*) suggesting that self-inhibitory effects of the preparation are unlikely. Based on initial velocities, peptide hydrolysis activities were computed at six different concentrations in the three cell fractions for the two dipeptides. The complete data are too extensive to be included but kinetic analysis of the data was undertaken as mentioned later.

The reproducibility of the in vitro assays (including in-

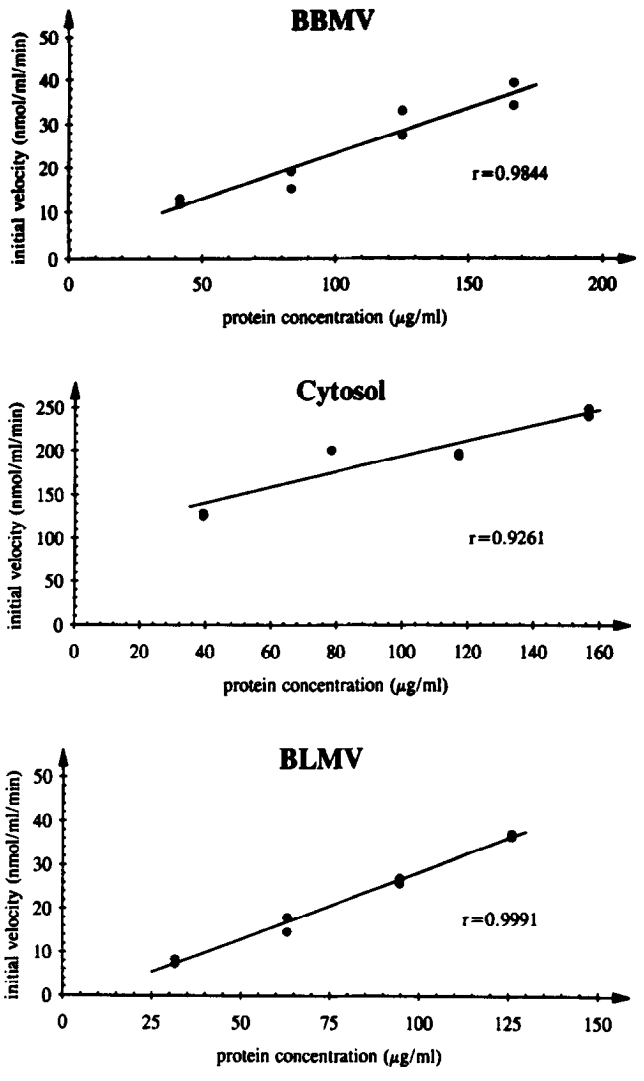

Figure 3 Linearity of initial velocity with protein concentration. The initial peptidase velocities of all cell fractions were measured at different protein concentrations in the reaction medium with excess of the substrate (Ala-Gln, 5 mmol/L). Each measurement was performed in duplicates.

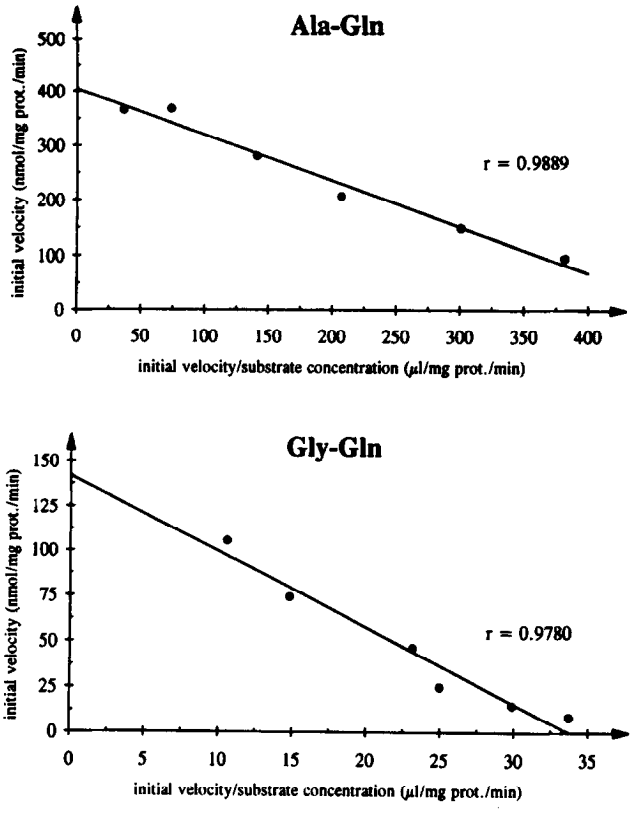

Figure 4 Eadie-Hofstee plot of BLMV. For calculation of the Michaelis constant and the maximum velocity the initial velocities were plotted against the quotient initial velocities/substrate concentration. The figure depicts the calculation of one BLMV preparation (12 incubations).

cubation procedure and analytics), was evaluated by repeated measurements of hydrolase activities against Ala-Gln (2 mmol/L; $n = 6$) of BBMV, SF, and BLMV within the same preparations. The initial velocities (nmol/(mg protein * min)) were 49.5 ± 2.4 in BBMV, 406.9 ± 53.8 in the SF, and 64.4 ± 4.8 in BLMV; the coefficients of variation were 5%, 13%, and 7% for brush border membrane, SF, and basolateral membrane, respectively.

Kinetic evaluation

The Eadie-Hofstee plots for the incubation of BLMV, BBMV, and SF with Ala-Gln and Gly-Gln all revealed high correlation coefficients suggesting that the data were robust with little variability. These results confirm that the peptide hydrolysis follows Michaelis-Menten kinetics. The Eadie-Hofstee plots for the incubation of BLMV are illustrated in *Figure 4* and the complete kinetic data are summarized in *Table 1*. The estimated K_m values for Ala-Gln appear to be lower in the three investigated cell fractions than those for Gly-Gln; the difference being less pronounced for the soluble fraction. Ala-Gln was equally accepted by the enzymes of BBMV, BLMV, and SF; but in contrast, for Gly-Gln, soluble peptidases showed a lower K_m suggestive of a higher affinity than those of membrane-bound enzymes. The capacity for hydrolysis, V_{max}, was higher for Ala-Gln than for Gly-Gln in all preparations of the different cell compartments, especially in the soluble fraction (*Table 1*).

Discussion

Results derived from previous investigations of peptide hydrolase activity in tissue homogenates are difficult to evaluate because of great variations in methodologies. A matter of particular concern could be the use of unphysiological experimental conditions that might erroneously influence the results. In the present study, great efforts were made to control and standardize the methodology with regard to incubation conditions (i.e., thawing and pretreatment of the samples) as well as suitable pH, temperature, and buffer composition. A similar pH of 7.4 in all incubations allowed a direct comparison of dipeptidase activities in BBMV, SF, and BLMV. The use of rp-HPLC with precolumn derivatisation[29] facilitated a sensitive and reliable analysis of substrates and products in the incubation mixture. These combined efforts are well-mirrored in the good reproducibility

Table 1 Kinetic constants of peptidase activity in mucosal cell fractions (mean ± SD, $n = 4$)

	Ala-Gln		Gly-Gln	
	K_m (mmol/L)	V_{max} (nmol/(mg prot * min))	K_m (mmol/L)	V_{max} (nmol/(mg prot * min))
BBMV	0.99 ± 0.23	610.8 ± 373.0	4.59 ± 2.01	125.4 ± 47.3
SF	0.82 ± 0.35	1784.5 ± 1041.0	2.24 ± 0.84	313.2 ± 107.8
BLMV	1.02 ± 0.38	342.2 ± 99.4	4.80 ± 0.89	122.6 ± 48.3

of the in vitro assay with coefficients of variations ranging from 5 to 13%. The isolated BBMV showed sufficient purity as evidenced by the 15-fold enrichment of the marker enzyme LAP. Similar enrichments were seen by previous investigators in isolated BBMV from rat[30] and dog intestine.[20] In good agreement with earlier studies reporting 10- to 17-fold enrichment of phosphatase activity we found 12- to 16-fold enrichment indicating acceptable purity of the BLMV preparation.[25,31,32]

All investigated cell fractions showed hydrolase activity towards Ala-Gln and Gly-Gln (*Figures 1* and *2*), suggesting that using the peptides as a glutamine source for the intestinal epithelial cells could occur in vivo. The time course of the incubations as well as the kinetic data (*Table 1*) strongly indicate higher affinity and capacity for Ala-Gln than for Gly-Gln, especially when considering the membrane-bound peptidases. Interestingly, in a previous paper[33], structure-related differences of peptidase activity in guinea-pig intestine against several dipeptides were observed. In line with the findings in the present study, the activity in both brush border and in the SF was considerably higher against the alanyl dipeptide than against the glycyl dipeptide.[33]

In the present study, characterization of the peptidase activity was attempted by assessing K_m and V_{max} of the hydrolysis (*Table 1*). We obtained virtually similar K_m-values in BBMV and BLMV for each peptide. This result suggests similar characteristics of the peptidases in both membrane fractions investigated. Indeed, the Michaelis constant is independent of the concentration of the enzyme and therefore can be used to evaluate enzyme affinities even in unpurified preparations and crude homogenates. The only prerequisite is that self-inhibitory effects of the preparations are excluded.[28]

Generally, a true comparison of V_{max}-values is only feasible with purified enzymes by referring V_{max} to the amount of the enzyme. However, tissue preparations and cell fractions consist of a complex mixture of enzymes and, thus, V_{max} by necessity is usually expressed per protein content. This invalidates comparison of the results from different studies, but the error arising from mixed protein preparations might be minimized if comparative evaluations of the respective V_{max}-values are made within each preparation. Thus, the 3- to 5-fold higher V_{max} for Ala-Gln (*Table 1*) can be interpreted as a tendency of higher capacity towards the alanylpeptide.

In the present study, employing rat BBMV, we measured a hydrolysis activity of 555 and 78 nmol/(mg protein * min) for Ala-Gln and Gly-Gln at 5 mmol/L substrate concentration. In a recent study, Minami et al.[34] assessed activities against glutamine-peptides using human intestinal mucosal BBMV. They observed hydrolysis rates for Ala-Gln and Gly-Gln of 1450 nmol/(mg protein * min) and 440 nmol/(mg protein * min), respectively. The higher activity in human mucosa specimen can only partly be explained by a higher enrichment of BBMV (20-fold, as assessed by sucrase activities). Presumably, part of the differences might be due to variations in incubation as well as analytical procedures. Minami et al.[34] determined the hydrolase activity in Tris buffer (pH 8) and analyzed amino acid and peptide concentrations by an ion-exchange chromatographic method using post column ninhydrin detection. Indeed, it is known that the pH optimum of isolated dipeptidases is near pH 8[22,35] and hydrolysis rates are slightly higher in Tris than in phosphate buffer.[36] Nevertheless, it might be concluded that the obvious differences in the hydrolase velocities against Ala-Gln and Gly-Gln are more pronounced in rat compared with human BBMV.

At least two mechanisms are discussed to contribute to dipeptide utilization by tissues:[37,38] transport of intact dipeptide across the cell membrane followed by intracellular hydrolysis or extracellular hydrolysis with absorption of the amino acids. Due to considerable dipeptidase activity, a distinction between transport capacity and extent of extracellular hydrolysis is very difficult, even in purified membrane vesicles. Thus in the majority of the peptide transport studies, hydrolysis resistant dipeptides like glycyl-proline and glycyl-sarcosine have been used.[32,39] Active dipeptide transport has been demonstrated in isolated BBMV from mouse and rabbit intestine[40,41] in renal BBMV from rabbit[42] and in enterocyte BLMV of rabbits.[32]

Investigations of possible assimilation of dipeptides in the liver, however provided no evidence of a peptide transport system, indicating extracellular hydrolysis by plasma-free or membrane-bound peptidases before transport of amino acids by conventional routes.[43] Similarly, Hundal et al. did not find a transport system for Ala-Gln but an extracellular peptidase activity in skeletal muscle.* These studies strongly indicate that under physiologic pH (7.4 to 8.0) intact peptide uptake plays a minor role in liver and muscle. Kinetic evaluation of the sarcolemmal peptidases against Ala-Gln and Gly-Gln revealed a comparable affinity as in the intestinal membranes, but a lower capacity.**

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The combined results of our study clearly indicate that dipeptide hydrolysis is catalyzed by membrane-bound peptidases found in brush border as well as in basolateral membranes. Because of the incubation conditions and analytic procedures used, we cannot distinguish whether the observed hydrolysis occurred extra- or intravesicularly. However, it is known that the microenvironment adjacent to the microvillious membrane is slightly acidic and that a proton gradient is the driving force for intact peptide transmembrane transport.[34,37,38,44] Because we do not employ pH (proton) gradient, it is unlikely that the glutamine dipeptides were actively transported.

In conclusion, the present study showed that intestinal mucosa is perfectly equipped to enable an efficient enzyme-catalyzed hydrolysis of glutamine-containing dipeptides either delivered luminally or intravenously. Accordingly, it has been repeatedly demonstrated in human and animal studies that provision of free glutamine or glutamine-containing dipeptides preserved intestinal integrity and enhanced mucosal cellularity and function.[45–47] The underlying mechanisms of glutamine utilization, however, remain to be elucidated.

Acknowledgments

This study was supported by Degussa AG (Hanau, Germany) and Fresenius (Bad Homburg, Germany). We are grateful to Professor M. Rennie (University of Dundee, U.K.) for valuable discussion.

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