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Research Paper

PEPT1 Enhances the Uptake of Gabapentin via Trans-Stimulation of $b^{0,+}$ Exchange

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Purpose. The aims of this study were (1) to determine whether amino acid and dipeptide loading can improve the effective permeability of gabapentin and (2) to characterize the underlying mechanism that is responsible for this interaction.
Materials and Methods. An *in situ* single-pass rat intestinal perfusion model was used to assess the effective permeability of gabapentin in rat, in the absence and presence of cellular loading by amino acid and dipeptide mixtures.
Results. Compared to gabapentin alone, cellular loading with amino acid and dipeptide mixtures significantly improved the effective permeability of gabapentin by 46–79% in jejunum and by 67–72% in ileum ($p \leq 0.01$). However, coperfusion of glycylsarcosine (i.e., PEPT1 substrate), methionine sulfoximine (i.e., glutamine synthase inhibitor), or lysine and arginine (i.e., $b^{0,+}$ substrates) with the amino acid and dipeptide mixtures compromised the intestinal uptake of gabapentin.
Conclusions. These findings demonstrate, for the first time, a direct relationship between the PEPT1-mediated uptake of a dipeptide and the trans-stimulated uptake of gabapentin (an amino acid-like drug) through the transport system $b^{0,+}$.

KEY WORDS: amino acid transporter $b^{0,+}$; gabapentin; intestinal perfusion; peptide transporter PEPT1; trans-stimulation.

INTRODUCTION

Gabapentin (Neurontin®) treats a host of CNS disorders including seizure, neuropathic pain, and anxiety with mechanism of action most likely at the $\alpha_2\delta$ subunit of a voltage-dependent Ca^{2+} channel (1–3). In addition to being zwitterionic at physiological pH, gabapentin has a small molecular weight of 171, low partition coefficient (log $D=-1.1$) and high water solubility (>100 mg/ml) (2). In humans and rats, it is not systemically metabolized and displays minimal or no protein binding (2). Structurally, it is related to the inhibitory neurotransmitter γ-amino butyric acid (GABA) and designed to improve GABA lipophilicity for increased access to the central nervous system (4). However, gabapentin permeation across the blood brain barrier remains largely mediated by the large neutral amino acid transporter, system LAT1 (5,6).

Transporters have also been implicated in the intestinal absorption of gabapentin. The intestinal epithelium possesses a host of carrier systems that facilitate the transport of neutral, cationic and anionic amino acids (7). Accordingly, the apical brush border membrane expresses the proton-dependent system PAT1, the sodium-dependent systems B^0, $B^{0,+}$, X^-_{AG}, ASC, and the sodium-independent system $b^{0,+}$. In contrast, the basolateral membrane contains the sodium-dependent systems A, N, y^+L, and the sodium-independent system LAT2. Our assessment in the rat small intestine indicated that gabapentin transport is both a proton- and sodium-independent process (see "Results" section). Consequently, all of the proton and sodium-dependent carriers at the apical and basolateral membranes can be ruled out as proteins associated with gabapentin intestinal transport. As a result, systems $b^{0,+}$ and LAT2 at the apical and basolateral membranes, respectively, are the carriers most likely responsible for the directional uptake of gabapentin from the intestinal lumen into the system circulation.

It has been shown that gabapentin possesses dose-dependent bioavailability (8) which is highly indicative of saturation of the carriers. Furthermore, gabapentin uptake has exhibited cross-inhibition with both cationic substrates such as arginine (9), and neutral amino acids such as leucine and phenylalanine (8,10,11), all of which are known substrates of both $b^{0,+}$ and LAT2. Evidence in rat brush border

This article is posthumous for David Fleisher

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ABBREVIATIONS: ala, alanine; arg, arginine; GABA, γ-amino butyric acid; gln, glutamine; glu, glutamate; GlyGlu, glycylglutamate; GlyGly, glycylglycine; GlySar, glycylsarcosine; GS, glutamine synthase; leu, leucine; lys, lysine; MS, methionine sulfoximine; P_{eff}, effective permeability; SLC, solute carrier family.

membrane vesicles have also demonstrated the sodium-independent uptake of gabapentin (9), further confirming the involvement of $b^{0,+}$ and LAT2. Both systems $b^{0,+}$ and LAT2 are exchangers belonging to the solute carrier families 3 and 7 (SLC3 and SLC7) (12,13). They facilitate the sodium-independent exchange of both cationic and neutral amino acids across the apical membrane ($b^{0,+}$) and neutral amino acids across the basolateral membrane (LAT2).

Wenzel *et al*. (14) were the first investigators to characterize the functional interaction between the uptake of free amino acids and peptides at a cellular level. They were able to demonstrate in Caco-2 cells how PEPT1-mediated uptake of dipeptides, and the subsequent hydrolysis to free amino acids, was capable of trans-stimulating the intestinal absorption of other amino acids via transport by system $b^{0,+}$. Beyond their work, there is limited information on this type of interaction. It is even less clear whether the same kind of relationship can be extrapolated to the intestinal uptake of dipeptides and amino acid-like drugs. We hypothesized that dipeptide uptake through PEPT1 can enhance the oral permeability of amino acid-like drugs, such as gabapentin, through a $b^{0,+}$-mediated trans-stimulation mechanism. In the present work, we used the *in situ* single-pass rat intestinal perfusion model to investigate whether a relationship exists between dipeptide uptake and gabapentin transport. Our main objectives were to assess the extent to which dipeptide loading changes the effective permeability (P_{eff}) of gabapentin, and to explore the mechanism responsible for this interaction.

MATERIALS AND METHODS

Chemicals

The uptake buffer consisted of 10 mM MES/Tris, 135 mM NaCl, 5 mM KCl, and 0.01% (w/v) PEG 4000 with pH adjusted to 7.4 or 6.0 by modifying the MES/Tris ratio. For sodium-free studies, sodium chloride was replaced with equimolar concentrations of choline chloride. All chemicals used in the buffer were purchased from Sigma-Aldrich (St. Louis, MO). Amino acids [alanine (ala), leucine (leu), glutamine (gln), glutamate (glu), arginine (arg), lysine (lys)] and dipeptides [glycylglutamate (GlyGlu), glycylsarcosine (GlySar), and glycylglycine (GlyGly)] were acquired from Sigma-Aldrich and Bachem (Torrance, CA), respectively. When used for cellular loading or inhibition experiments, the amino acid and dipeptide concentrations were fixed at 20 mM. Methionine sulfoximine (MS) was obtained from Sigma-Aldrich and used at 10 mM concentrations in these experiments. Unlabeled and [^{14}C]-labeled gabapentin were obtained from Sigma-Aldrich and Perkin Elmer (Wellesley, MA), respectively. For perfusion studies, the uptake buffer contained 10 μM of unlabeled gabapentin with 0.01 μmol of the [^{14}C]-labeled drug (sp. act. = 52 mCi/mmol).

Animal Surgery and Preparations

Male Sprague–Dawley rats weighing 280–360 g were acquired from Charles River Laboratory (Wilmington, MA) and fasted overnight (12–18 h, water ad libitum) prior to each experiment. In preparation for surgery, rats were sedated by intramuscular injection of a ketamine/xylazine cocktail (50 mg/kg and 10 mg/kg, respectively) and anesthetized with pentobarbital (40 mg/kg) by intraperitoneal injection. Following anesthesia, a 1 in. midline incision was made in the abdomen and the jejunal and ileal segments were prepared 3 cm distal to the ligament of Trietz and 3 cm proximal to the ileo–caecal junction, respectively. Small incisions were made at both ends of the 10 cm segments and the lumen was rinsed with warm normal saline. Both ends were cannulated with polyethylene tubing (i.d. 0.3 cm) and secured with thread sutures. To prevent dehydration, the intestinal segments were covered with gauze and wetted with normal saline. Following surgery, the animals were placed in a perfusion chamber (31°C), with the inlet tubing connected to a syringe pump (Harvard Apparatus, South Natick, MA) and the outlet tubing placed in a collection vial. Animals were sacrificed at the end of the experiment before recovering from anesthesia. All studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH publication No. 85-23, revised in 1985).

Trans-Stimulation Studies

For the trans-stimulation studies, gabapentin (10 μM) was perfused either (a) alone (control), (b) with an inclusive mixture (inclusive mix) containing both amino acids (ala, leu, gln, and glu at 20 mM each) and dipeptide (GlyGlu, 20 mM), (c) with only one dipeptide (GlyGlu, 20 mM), or (d) with only one amino acid (glu, 20 mM). For the control study, gabapentin was perfused through the intestinal segments for 120 min at a constant flow rate of 0.14 ml/min with the direction of flow from proximal to distal intestine. Perfusion experiments lasted no more than 120 min in order to preserve the integrity of the intestinal membrane.

To investigate the effect of the inclusive mixture on gabapentin P_{eff}, the study was carried out with an initial 30-minute preloading perfusion with the inclusive mix, followed by a 90-minute coperfusion of gabapentin with the same mixture without ala, leu, and gln. This experimental design was intended to impart the most favorable condition for observing the trans-stimulation of gabapentin uptake by amino acid and dipeptide substrates. To obtain maximal cellular loading, the preloading substrates were chosen for their ability to be transported into the intestinal epithelia by the brush border carriers (i.e., $b^{0,+}$, B^0, $X^-_{A,G}$, and PEPT1) and subsequently serve as intracellular substrates for $b^{0,+}$ exchange. Therefore, the neutral amino acids, ala, leu, and gln were preloaded into the cells by systems $b^{0,+}$ and B^0, while $X^-_{A,G}$ and PEPT1 facilitated the uptake of glu and GlyGlu, respectively. $X^-_{A,G}$ (SLC1) is a sodium- and proton-dependent, high-affinity glutamate transporter (15). PEPT1 (SLC15), on the other hand is a proton-dependent, oligopetide transporter that mediates the uptake of di- and tripeptides in the intestine (16). Following preloading, gabapentin was coperfused with only glu and GlyGlu, while ala, leu, and gln were omitted in order to avoid competition with gabapentin for uptake by $b^{0,+}$.

In addition to the inclusive mixture, glu and GlyGlu were also evaluated separately to assess their individual

influences on gabapentin absorption. Similarly, this was performed with a 30-minute preloading with either glu (20 mM) or GlyGlu (20 mM) followed by a 90-minute coperfusion of each respective substrate with gabapentin (10 μM).

Inhibition Studies

To characterize the mechanism by which amino acids and dipeptide trans-stimulate gabapentin uptake, inhibitors (i.e., 20 mM of either GlySar, MS, or lys/arg combination) were added to either the GlyGlu or the inclusive mixture solution and perfused through the rat intestinal segments. Again, similar to the trans-stimulation studies, the GlyGlu + inhibitor or inclusive mixture + inhibitor combination was preloaded for 30 min and then coperfused with the drug for 90 min. As a negative control, gabapentin permeability was also assessed with each of the inhibitors by itself.

All of the perfusion experiments were carried out in the presence of 0.01% (w/v) unlabeled PEG 4000 with 1 μmol of ^{3}H-PEG 4000 (sp. act. = 6 mCi/mmol). PEG 4000 is a large molecular weight compound incapable of permeation across the intestinal membrane. It was included in each experiment to assess whether loading conditions had generated a net water flux across the intestinal brush border membrane and consequently affecting gabapentin permeability (see "Data Analysis").

Analytical Methods

The perfusate exiting the outlet tubings were collected at 10 min intervals for 120 min. A 100 μl aliquot of each sample collection was added to 5 ml of scintillation cocktail (Ecolite, ICN, Costa Mesa, CA) and then counted on a Beckman LS 6000 liquid scintillation counter.

Data Analysis

Intestinal transport was determined from the steady-state loss of drug from the perfusate as it flowed through the intestine, which was achieved approximately 30–40 min after the start of perfusion. Lost drug was assumed to have permeated across the apical membrane into the mucosal epithelium by paracellular and/or transcellular mechanisms. The P_{eff} was calculated according to (17):

$$P_{eff} = -\ln[C_{out}/C_{in}]*[Q/2\pi rL]$$

where Q is the perfusion flow rate (0.14 ml/min), and $2\pi rL$ is the surface area across which gabapentin was absorbed with r as the radius of the rat intestine (0.18–0.3 cm) and L as the length of the perfused segments (10 cm). The C_{in} and C_{out} represent the concentration of drug entering and leaving the intestinal segments. C_{out} is corrected for water transport according to changes in the concentration of the nonabsorbable marker PEG 4000 (18):

$$C_{out} = C_{perfusate}/[PEG_{out}/PEG_{in}]$$

where $C_{perfusate}$ is the concentration of drug in the exiting perfusate, and PEG_{in} and PEG_{out} are the concentrations of PEG 4000 in perfusate going in and coming out, respectively, of the intestinal segment. The concentration of PEG 4000 fluctuated by about 1–3% which indicated that minimal water transport was evident during the experimental conditions.

Statistical Analysis

Data are reported as mean ± SE. To compare statistical significance between two groups, a two-tailed, paired *t*-test was performed. For multiple comparisons between different treatment conditions *versus* the control, a one-way ANOVA with Dunnett's post test was used. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (San Diego, CA). A probability of $p \leq 0.05$ was considered statistically significant.

RESULTS

Effect of pH, Sodium, and Basic Amino Acids on Gabapentin Transport

The intestinal transport of gabapentin was not significantly affected by the presence of protons (Fig. 1a) or sodium (Fig. 1b). These studies suggest the participation of the sodium and pH-independent apical and basolateral transporters, $b^{0,+}$ and LAT2, respectively, as the most likely carriers facilitating the directional uptake of gabapentin from the intestinal lumen into the system circulation. The dramatic impact of high affinity, basic amino acids substrates (i.e. lys and arg) in reducing gabapentin permeability further confirms the interaction of the drug with the apical $b^{0,+}$ transporter (Fig. 1c).

Effect of Loading with the Inclusive Mixture on Gabapentin P_{eff}

The presence of the inclusive mixture significantly increased gabapentin P_{eff} by 46 and 67% *versus* control in the jejunum and ileum, respectively (Fig. 2). Although it is possible that the neutral amino acids, ala, leu and gln in the preload could have influenced gabapentin transport, we believe the major contributing factors were glu and GlyGlu present during the 90-minute coperfusion. To assess which substrate was more influential on the intestinal permeability of gabapentin, each were loaded and evaluated separately. The results showed that GlyGlu enhanced gabapentin uptake in the jejunum and ileum by 79 and 72%, respectively, presumably through trans-stimulation of amino acid hydrolysis products. GlyGlu appeared to improve gabapentin transport in the jejunum to a greater extent than the inclusive mixture. Although speculative, it is possible that the microenvironment at the brush border membrane was not completely cleared of the neutral amino acid substrates. Thus, the small amounts of ala, leu, and gln still present may have competed with gabapentin for transport by $b^{0,+}$ and, thus, compromised the effect of the inclusive mixture. However, differences between the effect of GlyGlu alone and the inclusive mixture were small (<20%) and may reflect the variability typically observed with these types of experiments. In contrast to

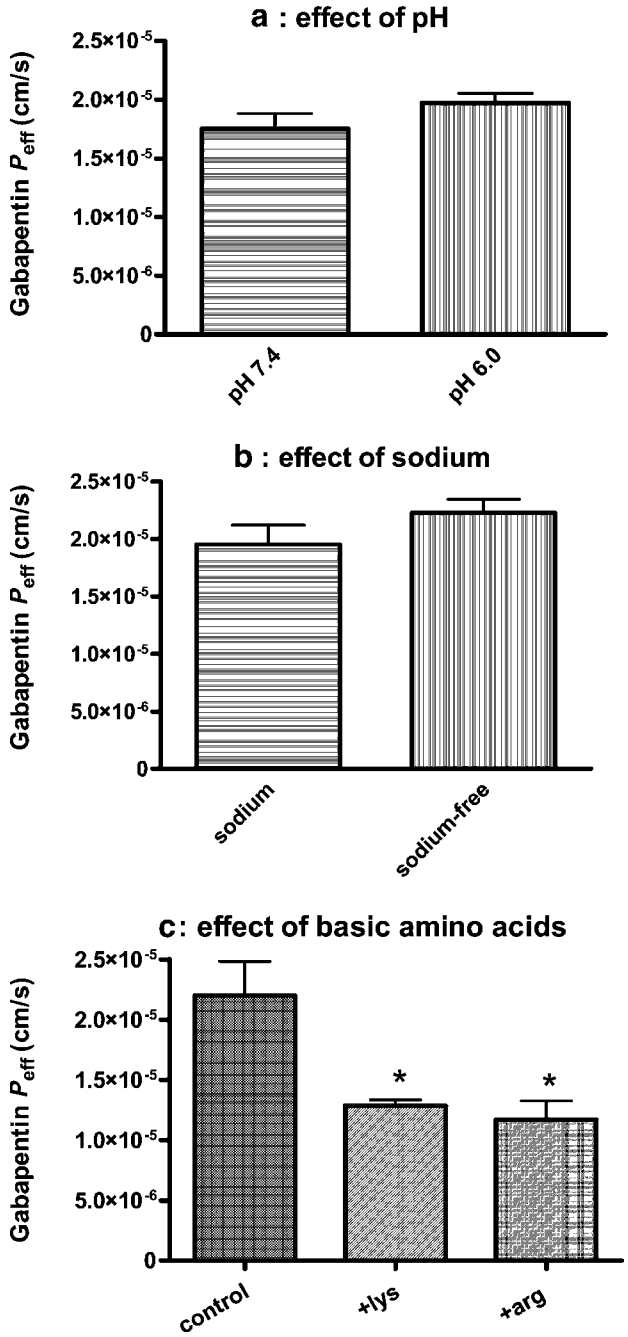

Fig. 1. Effect of pH (a), sodium (b), and basic amino acids (20 mM) (c) on the P_{eff} of gabapentin (10 μM) in rat small intestine. Data are reported as the mean ± SE ($n = 3 - 4$). *$p \leq 0.05$, as compared to control.

GlyGlu, glutamate alone did not have a significant effect on gabapentin uptake in either the upper or lower intestine. Thus, the evidence suggests that dipeptide loading was more effective than amino acids in enhancing gabapentin permeability.

Gabapentin P_{eff} in the Presence of Glucose or GlyGly

Nutrients such as growth factors, insulin, sugars and peptides (20,21) can enhance membrane expression of carrier proteins leading to increased activity. To investigate whether the increased gabapentin P_{eff} from GlyGlu was the result of a nutrient effect, 20 mM of glucose or GlyGly (two representative nutrients) were individually preloaded for 30 min and coperfused with 10 μM gabapentin for 90 min. The results showed neither substrate improved gabapentin P_{eff} in either the jejunum or ileum (Fig. 3), thus, removing any concerns of a nutrient effect on the improved permeability of gabapentin.

Assessment of Water Transport by Mannitol P_{eff}

Mannitol is a well known paracellular marker. Thus, its intestinal permeability would be greatly affected if the high

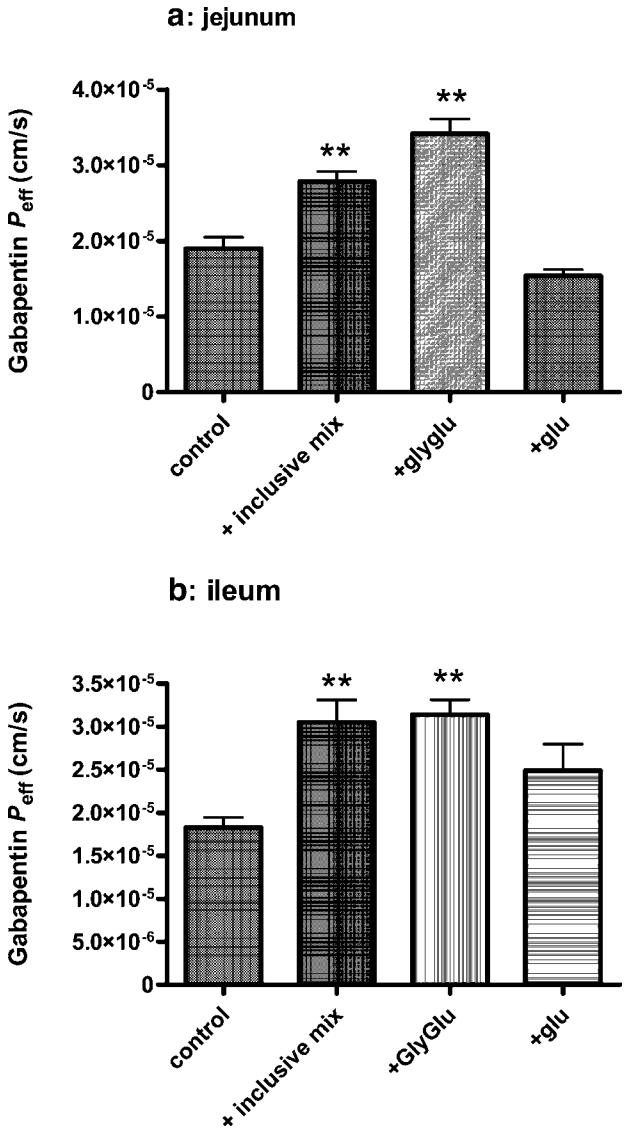

Fig. 2. Effect of amino acids and/or dipeptide loading on gabapentin P_{eff} in rat small intestine. The inclusive mixture (inclusive mix), glycylglutamate (GlyGlu), and glutamate (glu) (20 mM) were individually preloaded into rat jejunal (a) and ileal (b) segments for 30 min and then coperfused with gabapentin (10 μM) for 90 min. Data are reported as the mean ± SE ($n = 4 - 7$). **$p \leq 0.01$, as compared to control.

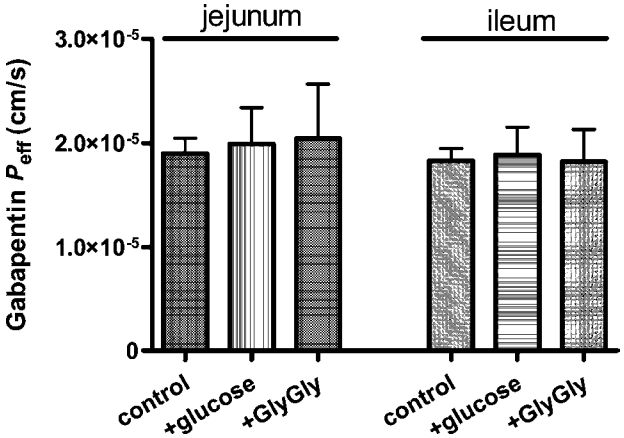

Fig. 3. Effect of glucose (20 mM) or GlyGly (20 mM) on the P_{eff} of gabapentin (10 μM) in rat small intestine. Glucose and GlyGly were individually preloaded in both the jejunum and ileum for 30 min and then coperfused with gabapentin for 90 min. Data are reported as the mean ± SE ($n = 4 - 7$).

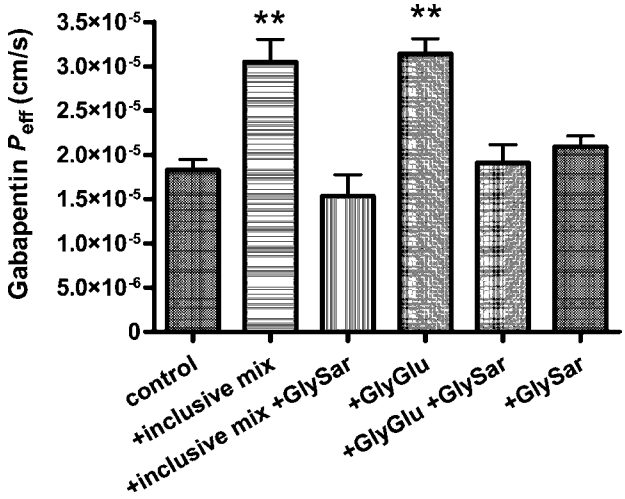

Fig. 5. Effect of GlySar on gabapentin P_{eff} in rat ileum. GlySar (20 mM) was preloaded for 30 min, either alone, with the inclusive mixture, or with GlyGlu (20 mM) and then coperfused for 90 min with gabapentin (10 μM). Data are reported as the mean ± SE ($n = 4 - 7$). $^{**}p \leq 0.01$, as compared to control.

solute load in the experimental design had generated either a significant water flux or a breach in the intestinal membrane integrity. To assess whether the loading conditions had compromised membrane integrity or produced substantial water transport, mannitol was perfused either alone or with the inclusive mixture. The results in Fig. 4 showed that amino acids and dipeptide loading had no effect on the P_{eff} of mannitol in either the jejunum or ileum. Therefore, the current experimental protocol did not compromise intestinal membrane integrity and was ruled out as a possible complicating factor in the uptake of gabapentin. Moreover, no significant changes in water transport were observed.

It should be noted that no differences were observed in gabapentin P_{eff} between jejunum and ileum for any of the treatment groups. This is consistent with studies by Howard *et al.*, (2004) showing equivalent expression of system $b^{0,+}$ mRNA in both the jejunum and ileum (19), thus confirming the region-independent transport of gabapentin. Thus, subsequent studies were performed only in the ileum.

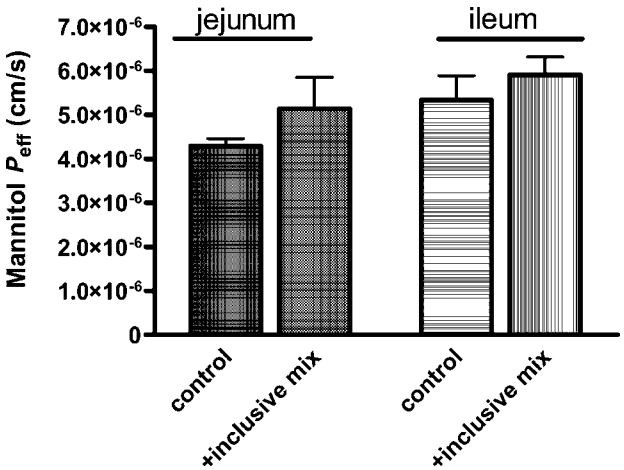

Fig. 4. Effect of amino acids and dipeptide inclusive mixture on mannitol P_{eff} in rat small intestine. The inclusive mixture (20 mM) was preloaded into rat jejunum and ileum for 30 min and then coperfused with mannitol (5 mM) for 90 min. Data are reported as the mean ± SE ($n = 4 - 8$).

Effect of GlySar on Gabapentin P_{eff}

GlySar is a hydrolysis-resistant dipeptide often used to assess PEPT1 transport activity. In rat ileum, GlySar (20 mM) was preloaded and coperfused with gabapentin alone, and appeared to have no influence on drug uptake (Fig. 5). As described previously, loading with the inclusive mixture or GlyGlu significantly enhanced the permeability of gabapentin. However, the inclusion of GlySar to the perfusate reduced the P_{eff} of gabapentin to levels not statistically different from control. Most likely, GlySar competed with GlyGlu for PEPT1 transport, thereby depleting the intracellular amino acid pool and compromising $b^{0,+}$ exchange of gabapentin with constituent amino acids of the dipeptide. Thus, PEPT1 uptake of GlyGlu was essential for improving the P_{eff} gabapentin.

Effect of Glutamine Synthase Inhibition on Trans-Stimulation of Gabapentin

Methionine sulfoximine (MS) is a specific, irreversible inhibitor of glutamine synthase (GS) (22,23). When preloaded and coperfused with gabapentin, MS (10 mM) alone did not influence drug uptake (Fig. 6). However, its addition to the dipeptide mixture resulted in GlyGlu loosing its positive influence on gabapentin permeability. Thus, the gabapentin P_{eff} was significantly decreased from 172 to 96% of control in rat ileum. This was most likely due to the inhibition of GS by MS, leading to glutamate accumulation instead of glutamine in the cellular compartment. Since glutamate is not a substrate for $b^{0,+}$ exchange, it would not impact gabapentin exchange. This finding supports the contention that glutamine

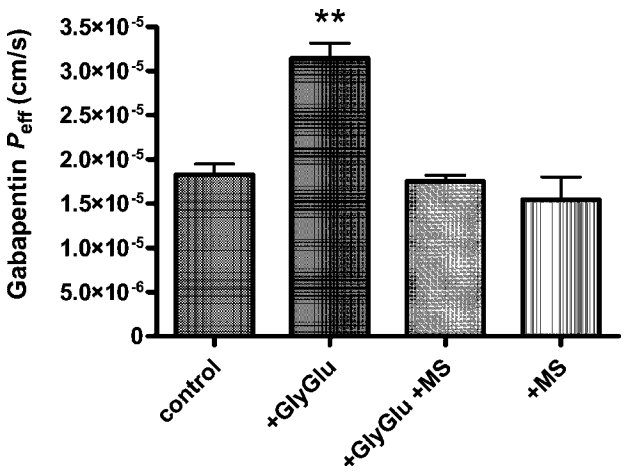

Fig. 6. Effect of methionine sulfoximine (*MS*) on gabapentin P_{eff} in rat ileum. MS (10 mM) was preloaded alone or with GlyGlu (20 mM) and then coperfused for 90 min with gabapentin (10 μM). Data are presented as the mean ± SE ($n = 4 - 7$). $^{**}p \leq 0.01$, as compared to control.

synthesis is another important step in the GlyGlu-mediated trans-stimulation of gabapentin uptake by $b^{0,+}$.

Effect of Lysine and Arginine on Gabapentin P_{eff}

Both arg and lys are high affinity, basic amino acid substrates for $b^{0,+}$ with K_m values ranging from 100–400 μmol/l (24). To maximize saturation of the $b^{0,+}$ transporter, competition studies were performed by incorporating both these amino acids (20 mM each) into the GlyGlu and inclusive mixture. Similar to previous results with GlySar and MS, the inclusion of lys and arg significantly reduced the elevated gabapentin P_{eff} to levels not statistically different from control (Fig. 7). This observation supports the view that

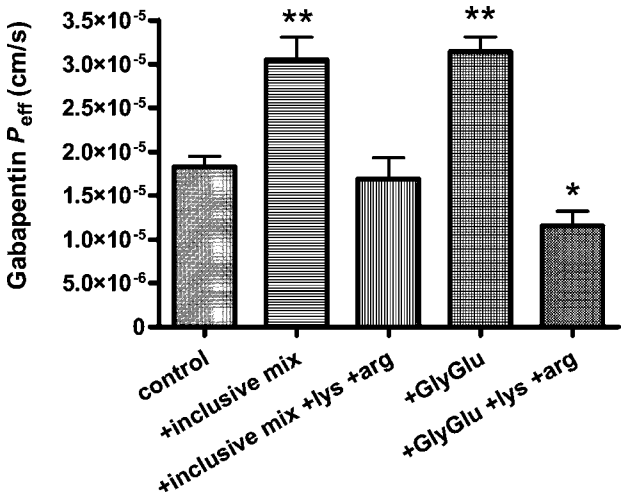

Fig. 7. Effect of lysine (lys) and arginine (arg) on gabapentin P_{eff} in rat ileum. The mixture of lys and arg (20 mM each) was preloaded for 30 min with the inclusive mixture or GlyGlu (20 mM) and then coperfused for 90 min with gabapentin (10 μM). Data are presented as the mean±SE ($n = 4 - 7$). $^{**}p \leq 0.05$ and $^{**}p \leq 0.01$, as compared to control.

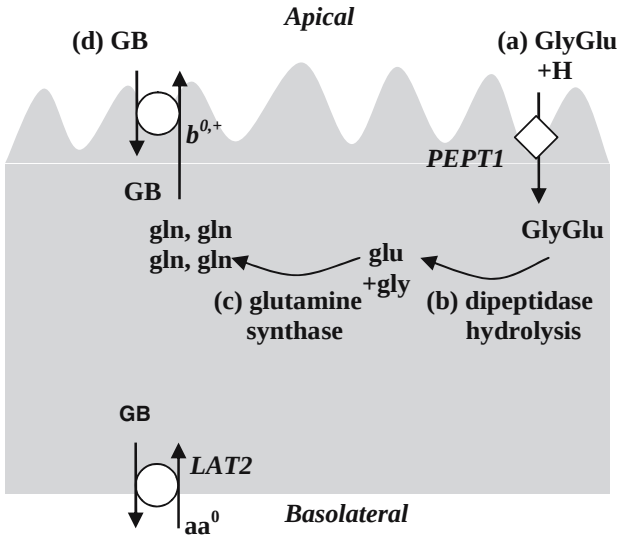

Fig. 8. Proposed mechanism for GlyGlu *trans*-stimulation of gabapentin (GB) uptake at the rat intestinal brush border membrane. The sequential steps in this process include: (a) PEPT1 uptake of GlyGlu, (b) dipeptidase hydrolysis to glycine (gly) and glutamate (glu), (c) conversion of glutamate to glutamine (gln) by glutamine synthase, and (d) trans-stimulation of gabapentin uptake via $b^{0,+}$-mediated exchange. At the basolateral membrane, system LAT2 facilitates the efflux of gabapentin into the systemic circulation through an exchange with extracellular neutral amino acids (aa^0).

$b^{0,+}$ exchange activity is critical for dipeptide-mediated trans-stimulation of gabapentin in the intestine.

DISCUSSION

Understanding the relationship between food effects from protein meals and drug bioavailability is crucial because of the implications on altered pharmacokinetics and therapeutic response. Moreover, a priori predictions of whether or not such interactions occur are difficult, as is the direction of change. In 1996, Gidal *et al.* (25) found that, unexpectedly, the maximum serum concentration of gabapentin in healthy human subjects was significantly increased by 36% when administered with a high-protein meal. Based on this finding, the authors speculated that the large amino acid load delivered with the high-protein meal might enhance gabapentin absorption via trans-stimulation of an amino acid carrier system. In 2001, Wenzel et al. (14) demonstrated in Caco-2 cells that the intracellular free amino acid concentration is the main determinant for the extent of trans-stimulation of amino acid absorption, but that dipeptide loading by PEPT1 was a more efficient process. Using a model system that more closely resembles *in vivo* absorption from the intestine, our studies demonstrate a direct interplay between dipeptide transport and enhanced gabapentin uptake. Based on our findings, the following mechanism has been proposed (Fig. 8). In this model, PEPT1 mediates the uptake of GlyGlu followed by intracellular hydrolysis to its constituent amino acids, glycine and glutamate. The latter amino acid is then converted to glutamine, which then provides the driving force for the trans-stimulated absorption of gabapentin via $b^{0,+}$ exchange.

The *in situ* single-pass rat intestinal perfusion model was used because it has many advantages over an *in vitro* cell-based model. Specifically, it provides experimental conditions that better reflect the *in vivo* environment encountered following oral drug administration. These include lower sensitivity to pH variations due to a preserved microclimate (26,27), the presence of an intact intestinal blood supply, and the presence of enzymes and brush border transporters (28). Moreover, the expression of metabolic enzymes and transporters are differentially expressed along the intestinal tract (19,29–32) and, as a result, perfusion studies can be performed at different intestinal segments in order to evaluate the impact of region-dependent luminal drug uptake. Finally, the effective permeabilities from rat perfusion studies highly correlate with the extent of oral drug absorption in humans (28,33–35).

Cellular loading of rat intestinal epithelia with the inclusive amino acid and dipeptide mixture significantly increased the P_{eff} of gabapentin in both the jejunum and ileum. However, assessment of the individual components within the mixture revealed that unlike glutamate, GlyGlu was able to significantly improve the absorption of gabapentin. At first glance, the glutamine pool, whether it was generated from glutamate that was absorbed as free amino acid or hydrolyzed from GlyGlu, should have produced similar effects on gabapentin P_{eff}. However, these results are consistent with the evidence supporting a more rapid rate of dipeptide absorption than that of corresponding amino acids (16,36). Thus, it is likely that GlyGlu loading was more efficient than that of glutamate, which lead to better *trans*-stimulation of $b^{0,+}$ exchange and therefore higher drug uptake.

The specificity of GlyGlu on gabapentin transport, as compared to dipeptides in general, was also explored (i.e., can the hydrolysis products of any intracellular dipeptide enhance gabapentin uptake?). The lack of effect by GlyGly suggests that the mechanism underlying dipeptide trans-stimulation of gabapentin absorption is more specific than simply using a PEPT1-mediated substrate. Precisely, it must include substrates that are capable of being hydrolyzed to neutral or cationic amino acids which can then be utilized by $b^{0,+}$ for exchange with extracellular drug. In theory, dipeptides containing arginine, lysine, alanine, leucine, and glutamine should work. However, there was concern that these dipeptides might create a microenvironment that competes with drug for $b^{0,+}$ uptake since approximately 10% is metabolized at the intestinal brush border membrane. This was observed when 20 mM of GlyArg inhibited gabapentin permeability to the same extent as 20 mM of arginine (data not shown). To circumvent this problem, GlyGlu was studied because brush border hydrolases would generate an extracellular microenvironment of free glycine and glutamate. Both of these amino acids are not substrates of $b^{0,+}$ and, therefore, would be unable to compete with gabapentin for uptake. Moreover, intracellular glutamate has to be converted to glutamine before it can interact with the $b^{0,+}$ exchanger.

Impeding intestinal intracellular peptidases would also lead to a reduced glutamine pool. Given their diversity and broad substrate specificity (37), the complete inhibition of GlyGlu hydrolysis would be logistically impractical. For this reason, inhibition of peptide hydrolase was omitted in the present study. However, with approximately 80–90% of the intestinal peptidases residing intracellularly (37,38), we are confident that the fate of GlyGlu, once absorbed, would involve hydrolysis. This belief is consistent with the intracellular conversion of glutamate to glutamine, as demonstrated with the methionine sulfoximine inhibition studies.

In conclusion, our studies demonstrate, for the first time, a direct relationship between the PEPT1-mediated uptake of a dipeptide and the trans-stimulated uptake of gabapentin (an amino acid-like drug) through the transport system $b^{0,+}$. Future studies will be directed at determining whether or not such an interaction occurs *in vivo*.

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