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Kinetic measurements of di- and tripeptide and peptidomimetic drug transport in different kidney regions using the fluorescent membrane potential sensitive dye, DiS-C_3-(3)

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Abstract

Tri- and dipeptide are transported in the kidney by PEPT1 and PEPT2 isoforms. The aim of this study was to investigate differences in transport kinetics between renal brush border (BBMV) and outer medulla (OMMV) membrane vesicles (where PEPT1 and PEPT2 are sequentially available) for a range of di- and tripeptides and peptidomimetic drugs. This was accomplished through the use of the potential sensitive fluorescent dye 3,3'-dipropylthiacarbocyanine iodide [DiS-C_3-(3)]. BBMV and OMMV were prepared from rat kidney using standard techniques. The presence of PEPT1 in BBMV and PEPT2 in OMMV was confirmed using Western blotting. Fluorescence changes were measured when extra-vesicular media at pH 6.6 containing 0-1 mM substrates was added to a cuvette containing vesicles pre-equilibrated at pH 7.4 and 2.71μM DiS-C_3-(3). An increase in fluorescence intensity occurred upon substrate addition reflecting the expected positive change in membrane potential difference. Of the range of substrates studied, OMMV manifested the highest affinity to cefadroxil and Valacyclovir (K_m 4.3 ± 1.2 and 11.7 ± 3.2μM respectively) compared to other substrates, whilst the BBMV showed a higher affinity to Gly-His (K_m 15.4 ± 3.1μM) compared to other substrates. In addition, OMMV showed higher affinity and capacity to Gly-Gln (K_m 47.1 ± 9.8μM, 55.5 ± 2.8 ΔF/S/mg protein) than BBMV (K_m 78.1 ± 13.3μM and 35.5 ± 1.7 ΔF/S/mg protein respectively). In conclusion, this study successfully separated the expression of PEPT1 and PEPT2 into different vesicle preparations inferring their activity in different regions of the renal proximal tubule.

Key Words: DiS-C_3-(3); PEPT co-transporters; renal membrane vesicles.

Introduction

The generation and regulation of a membrane potential difference is a dynamic process and fundamentally crucial to many physiological functions. For example, the energy released from ATP hydrolysis is used by the Na^+, K^+ ATPase to transport three Na^+ ions out of the cell in exchange for two K^+ ions pumped into the cell (Blostein 1999). This, in combination with the passive potassium and chloride fluxes creates a negative membrane potential difference that favours the entry of sodium ions. This electrochemical gradient generates the driving force that facilitates the efficient absorption of many nutrients and some important drugs (Faller 2008; Jorgensen 1986). The reabsorption of amino acids in the kidney proximal tubule is a vital process to the body's protein balance. Small peptides in the form of 2-3 amino acids are transported by the H^+-dependent transporters PEPT1 (low-affinity/high-capacity) and PEPT2 (high-affinity/low-capacity) (Daniel and Kottra 2004). The translocation of di- and tripeptides by these cotransporters is forced by a transmembrane electrochemical proton gradient. This is mainly facilitated by the apical Na^+-H^+ exchanger (NHE3), which acidifies the lumen to around pH 6.7-6.8 by continuously secreting H^+ across the brush border membrane (Daniel and Kottra 2004; Vieira and Malnic 1968). The resultant H^+ inward electrochemical gradient can then be utilised by PEPT cotransporters to force the uptake of any of the naturally-occurring 400 dipeptide and 8000 potential tripeptide combinations into the proximal tubule cells (Anderson et al. 2010; Rubio-Aliaga 2008). PEPT transporters are also capable of transporting a vast range of peptidomimetic drugs including β-lactam antibiotics of the aminocephalosporin (e.g. cefadroxil) and Penicillin (e.g. ampicillin) classes; some antiviral (e.g. valacyclovir); and anti-tumour drugs (e.g. δ-amino-levulinic acid and γ-glutamyl-$_L$-cysteine); and selected angiotensin converting enzyme (ACE) inhibitors (e.g. captopril) (Brandsch et al. 2008).

PEPT1 and PEPT2 transporters are heterogeneously distributed in the renal proximal tubule, as PEPT1 is confined to segment 1, while PEPT2 is restricted predominantly to the later S3 segments (Shen et al 1999). PEPT2 was found in higher quantity than PEPT1 (Leibach and Ganapathy 1996; Smith et al 1998), hence it was suggested that PEPT2 is the main cotransporter for the uptake of peptides and peptidomimetic drugs (Shen et al. 1999). Several studies using either transfected cell lines with PEPT1 and PEPT2 (Terada et al 1997) or BBMV (Ries et al 1994; Takahashi et al 1998) revealed that β-lactam antibiotic containing an α-amino group (e.g cefadroxil) had much higher affinity to the renal PEPT2. However, the relative contribution of the native PEPT1 and PEPT2 isoforms in transporting oligopeptides and peptidomimetic drugs in the renal proximal tubule is not clear yet. It was suggested that further studies investigating the distribution and expression level of PEPT1 and PEPT2 and also their contribution to the tubular reabsorption are needed for differentiating between their roles (Takahashi et al 1998).

In the past BBMV have been a popular method for studying the characteristics of di- and tripeptide transport by PEPT1 and PEPT2 (Daniel et al 1991; Ries et al 1994; Takahasi et al 1998). This is partly because the absence of metabolic machinery allows the accumulation of the intact substrate in the intra-vesicular space to be measured. Traditionally, such experiments have involved the measurement of radioisotope flux (Daniel et al 1991; Ries et al 1994; Takahasi et al 1998) or DiS-C_3-(5) (Plášek et al 1994). We have developed a method of measuring the kinetic parameters for the uptake of some di- and tripeptide and selected peptidomimetic drugs into renal BBMV and OMMV via the use of the fluorescent potential sensitive dye, 3,3'-dipropylthiacarbocyanine iodide [DiS-C_3-(3)]. In a previous publication we showed that DiS-C_3-(3) could be used to detect changes in membrane potential difference

in renal membrane vesicles but did not measure the kinetics of substrate transport (Alghamdi and King 2012). The reason why it is important to measure the transport kinetics in different regions of the kidney is predicting the bioavailability of different substrates and drugs or where genetic variations exist in the transporters and their expression.

Materials and Methods

Chemicals and Kidneys

All chemicals and reagents used in this study were obtained from SIGMA, Chem-Supply AppliChem, Santa Cruz Biotechnology, or GE Healthcare and were of analytical quality. Kidneys were obtained from male Wistar rats of 6-7 months old, which were sacrificed by stunning and cervical dislocation. The kidneys were snap frozen in liquid nitrogen and then transferred to a -80°C freezer until used for the preparation of BBMV and OMMV. This study was approved by the Animal Ethics committee of the University of New England, and complies with the *Guide for the care and use of laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Isolation of BBMV and OMMV and quality control

The BBMV and OMMV were isolated by the two-step Mg^{++} /EGTA precipitation method (Biber et al 1981; Brown et al 1993; Daniel et al 1991). BBMV were prepared from ~0.5-1 mm thick slices of superficial cortex, while OMMV were prepared from slices of outer medulla, which are characterised by having red stripes (Kragh-Hansen and Shiekh 1984; Smith et al 1998). The integrity of the purified vesicles was examined using marker enzyme

assays. The enrichments of alkaline phosphatase and leucine aminopeptidase for BBMV were 13.51 ± 2.07 and 12.82 ± 1.32 fold respectively, and for OMMV were 11.3 ± 0.75 and 17.23 ± 2.4 fold respectively. These values of enzyme enrichments indicate good, non-contaminated vesicles (Biber et al 1981). Protein concentration was quantified using Bradford's method (Brown et al 1993).

After isolating the vesicles and before placing them in the -80°C freezer, they were suspended in buffer 1 (intra-vesicular medium) containing (in mM): 100 mannitol, 100 KH_2PO_4 and 10 HEPES (pH 7.4 with TRIS) and centrifuged at 31,000 x g for 20 min at 4°C (Daniel et al 1991). The supernatant was discarded and the pellet was re-suspended with the same solution and pre-equilibrated using freeze-thaw fractionation (Donowitz et al 1987). The transport buffer (buffer 2) (extra-vesicular medium) contained (in mM): 100 mannitol, 100 KH_2PO_4, and 10 2-(N-Morpholino) ethanesulfonic acid (MES, pH 6.6 with TRIS).

Western blot analysis

As PEPT1 and PEPT2 are located in different segments of the kidney (superficial cortex and outer medulla, respectively) (Shen et al 1999), Western blot was used to confirm the positive/negative expression in the isolated BBMV and OMMV. BBMV and OMMV containing 45µg protein/sample were mixed in eppendorf tubes with the loading buffer (3X) containing 6% Sodium dodecyl sulfate (SDS), 0.3 % Bromophenol blue, 30% glycerol, 5% β-mercaptoethanol, and 150 mM Tris-HCl (pH 6.8). They were transferred into a boiling water bath at 100°C for 5 minutes. After that, the tube contents were loaded onto Mini-Protean Bio-Rad Gels 4-20 % and the first well was always loaded with 5 µl of Precision Plus Protein

Standards as a molecular weight control ladder. Gels were run at 120 V constant voltage for 40-90 minutes. The gels were then blotted with nitrocellulose membrane (semi-dry system) at 120 mA for 3 hours. The membranes were stripped for 15 minutes as described by Kaur, Bachhawat (2009) and incubated overnight with blocking buffer 5 % skim milk dissolved in TBS-T buffer containing (in mM) 150 NaCl, 20 Tris-HCl (pH 7.5) and 0.1 % Tween 20. The membranes were then incubated with either mouse monoclonal anti-PEPT1 for 1 h at room temperature (RT) or with goat polyclonal anti-PEPT2 overnight at 4°C. This was followed by an hour's incubation with HRP-conjugated goat anti-mouse and mouse anti-goat IgG respectively at RT. Between probing with the primary, and secondary antibodies and after incubation with the secondary antibodies, the membranes were washed 3 times for 10 min with TBS-T buffer. Protein bands were detected using ECL^{TM} Western Blotting Analysis System (GE Healthcare) according to the manufacturer's instructions. Scanned pictures of Hyperfilm ECL were taken using BIO-RAD GEL DOC EQ system. The membranes were transferred to a stripping buffer containing (in mM) 100 β-mercaptoethanol, 62.5 Tris-HCl (pH, 6.7), and 2% SDS at 55 C for 30 minutes, followed by 2 ×10 minute washes with TBS-T. Then, they were incubated with the blocking buffer and reprobed with anti-β-Actin (mouse monoclonal) antibodies, following the steps described above. This was used as a loading control and confirmed that each lane had equal amounts of protein loaded into it (results not shown).

Fluorescence Measurements of Substrate Transport

These measurements were carried out at (20-24°C) in a fluorescence spectrophotometer (Perkin-Elmer LS 55), pre-set with: 5nm slit width, excitation and emission wavelengths of 557 and 575nm respectively; and the cuvettes used had a pathlength of 2mm. Fluorescence

intensity (in arbitrary units) was recorded at 0.02 second intervals for about 80 seconds and displayed on a digital screen as a plot of fluorescence versus time (see Figure 2 for an example).

Experiments were started by mixing appropriate volumes of buffer 2, 4µl from a 541µM stock (2.71µM final concentration) DiS-C_3-(3), and sufficient vesicles to obtain a constant protein concentration of 45µg (maintaining a dye to protein ratio of 19.4µg/mg (Cassano et al 1988; Wright et al 1981) to a total volume of 800µl into an Eppendorf tube. This Eppendorf tube was gently mixed using a pipette. The total volume was then divided into two matched cuvettes (each one had 396µl), one was for the sample, whilst the other one served as control. Both of which were placed into the fluorimeter and the fluorescence followed for 50 seconds to enable stabilisation of the signal. A series of concentrations of the substrates were made as stock solutions so that a constant volume of 4µl were added for each sample, as the addition of different volumes could cause variations in the fluorescence intensity. After adding substrates (e.g. Gly-Gln), the fluorescence was measured for approximately another 50 seconds until the fluorescence had stabilised. Graphs of the fluorescence measured (in arbitrary units) vs. time (in 0.02s) were plotted as shown in Figure 2. In the control samples no substrates were added.

Data Analysis and Presentation:

The readings for the first period during which fluorescence was stable (approx. 50 seconds) were removed from all samples, as this period was used to check that the system was in the steady state. After that, all data were normalised and each sample was corrected for the

changes of fluorescence due to the addition of the substrate by subtracting the trace obtained for the corresponding control samples. Then, the average of these corrected readings for each concentration was plotted. The total change in fluorescence intensity was then calculated by subtracting the intensity at the start of the transport to the intensity at the plateau. This was then repeated for different substrate concentrations. The readings were then plotted against their corresponding substrate concentrations and fitted using a Michaelis-Menten curve (R^2 values which demonstrated a goodness of fit >90) and equation ($V = \frac{Vmax\,[S]}{Km+[S]}$), which was then used to calculate V_{max} and K_m. V_{max} values were calculated as ΔF/0.02s/45μg protein, which were converted to ΔF/s/mg protein. Student's t-Test was then calculated using these equations: $t = \frac{Difference\ between\ the\ two\ means}{s.e.of\ the\ difference}$ and $s.e.difference = \sqrt{\frac{^2_SA}{n_A} + \frac{^2_SB}{n_B}}$ to illustrate significant differences between kinetic values. All of the results presented are means ± SE of *n* = 6-8 samples. *P* values < 0.05 were considered to be statistically significant.

Results

Confirmation of the sequential distribution of PEPT1 and PEPT2 in BBMV and OMMV respectively

All vesicles used in this study were examined by Western blot to confirm the correct distribution of PEPT1 and PEPT2. PEPT1, PEPT2 and β-actin protein bands were detected in BBMV and OMMV with molecular weight of 75, 85 and 43 kDa, respectively. As shown in the upper panel of Figure 1, PEPT1 was present in the first 2 lanes, which were loaded with BBMV, while it was absent in lanes 3-11 where OMMV were loaded. The middle panel of Figure 1 shows that PEPT2 was absent in lanes 4-6 and 8-11 in which BBMV were loaded,

while PEPT2 bands were clearly shown in the lanes loaded with OMMV. The bottom panel of Figure 1 shows that the house-keeper, ß-actin was present in all samples.

Measurement of fluorescence changes as a function of time upon the addition of extra-vesicular medium with or without 1000µM Gly-Gln

Figure 2 shows the result of a single experiment representative of 7 such experiments for renal BBMV and OMMV. A sharp decrease in fluorescence was seen as soon as 1000µM Gly-Gln was added, followed by an increase and plateau with a largely unchanging fluorescence level that continued for the rest of the experiment. This general pattern was observed in all experiments when different concentrations of substrates were delivered to both BBMV and OMMV. When the same volume of extra-vesicular buffer containing no Gly-Gln was added to both BBMV and OMMV, there was a similar decrease in fluorescence followed by a plateau (Figure 2). This pattern of fluorescence was seen in all vesicles, except for at a low concentration of some substrates (10-50 µM) delivered to OMMV which showed a slightly smaller decrease in fluorescence than the control, followed by a sharp increase in fluorescence.

Kinetics of substrate uptake into BBMV and OMMV

Figures 3 to 5 compare the Michaelis-Menten curves for the transport kinetics of a range of substrates measured using an extra-vesicular pH of 6.6. When comparing the transport of Gly-Gln (circles, dashed line), Gly-His (squares, continuous line) and cefadroxil (triangles, bold dashed line) in BBMV (Figure 3), the V_{max} for Gly-His transport was significantly

higher than for Gly-Gln transport (57.3 ± 2.1 ΔF/s/mg protein and 35.5 ± 1.7 ΔF/s/mg protein respectively, P= 0.001). Similarly, the K_m for Gly-His transport was significantly lower than for Gly-Gln (15.4 ± 3.1μM and 78.1 ± 13.3μM respectively, P=0.002). cefadroxil (Figure 3) had an affinity for the transporters situated between Gly-His and Gly-Gln (34.1 ± 8.4μM) but with a much higher capacity (82.7 ± 5.67 ΔF/s/mg protein) than for the 2 dipeptides.

The same trend for the two dipeptides' transport was observed in OMMV (Figure 4) compared to BBMV, with a K_m significantly lower for Gly-His (squares, continuous line) transport than Gly-Gln (circles, dashed line) (12.5 ± 3.1μM and 47.1 ± 9.8μM respectively, P=0.012) and a decrease (although not significant) in V_{max} between Gly-His and Gly-Gln (61.1 ± 2.5 ΔF/s/mg protein and 55.5 ± 2.8 ΔF/s/mg protein respectively, P=0.1). However, and unlike the BBMV, cefadroxil had a significantly higher transporter affinity (4.3 ± 1.2μM) than both dipeptides (squares, continuous line; Figure 4). In addition, the K_m for Gly-Gly-His transport (triangles, dashed line) (47.5 ± 6.65μM) was significantly lower than for Gly-His transport (P=0.002) but similar to the Gly-Gln transport. However, the V_{max} for Gly-Gly-His transport (75.9 ± 2.33 ΔF/s/mg protein) was significantly higher than for Gly-His (P=0.0034) and Gly-Gln (P=0.0006) transport.

When comparing peptidomimetic drug substrates in OMMV, the K_m of cefadroxil was significantly lower than δ-amino-levulinic (P=0.001), but close to statistical significance with Valacyclovir (P=0.06; Figure 5). Also, the V_{max} was significantly higher for cefadroxil than for other two drugs (P=0.002 and 0.001 respectively, Table 1).

A complete list of the K_m and V_{max} for all substrates measured in the BBMV and OMMV is shown in table 1. This shows that the V_{max} for Gly-Gln transport in BBMV was significantly lower than the V_{max} for Gly-Gln (P=0.001) transport in OMMV. Also, the transport of Gly-Gln in OMMV has a higher affinity than in BBMV, although not significant (P=0.09). However, while the same trends were observed for V_{max} and K_m in the case of Gly-His, the differences between BBMV and OMMV were not significant. In addition, there was no significant difference in the V_{max} for cefadroxil transport between BBMV and OMMV, However the K_m for cefadroxil transport in OMMV was significantly lower than in BBMV (P= 0.001).

Discussion

The reabsorption of important nutrients in the kidney occurs mostly in the proximal tubule where the proton-dependent PEPT1 and PEPT2 cotransporters are located. The electrochemical gradient created by the inward cation and membrane potential difference is the main force driving these reabsorptive processes. Indeed, the transport of these substrates by PEPT1 and PEPT2 is well defined to be a proton-dependent process in which translocation of the substrate is accompanied by hydrogen ion. Measuring the membrane potential difference could provide important insights to the renal functions for nutrient handling, wastes, and also the pharmacokinetics of many drugs. Several studies have shown the possible applications of Cyanine dyes as a membrane fluorescent biosensor on BBMV (Beck et al 1978; Cassano et al 1988; Kragh-Hansen et al 1982; Ries et al 1994; Vayro and Simmons 1996; Wright et al 1981). Some studies used extracellular pH 6.5-6.8 (Daniel et al 1991; Kottra et al 2013) similar to the physiological level to create an inward H^+ gradient for activating theses transporters. By following these studies, the H^+ gradient was created by

adjusting the pH of the extra-vesicular and intra-vesicular medium to be 6.6 and 7.4 respectively (~ 6.7 across the membrane). This is based on the hypothesis that at these pH environments and with increasing concentration of substrates delivered to either BBMV or OMMV, corresponding increases in the fluorescence signal (positive membrane potential difference) would result from a greater influx of protons, which would reflect the translocation of the substrates (Figures 2 to 5). These changes in fluorescence are similar to that seen in the study conducted by Ries et al. (1994), however they used a different membrane potential sensitive dye DiS-C_3-(5), and they did not isolate different regions of the proximal tubule representing PEPT1 and PEPT2 (BBMV and OMMV respectively). Therefore, this prompted us to develop a method for measuring the membrane potential difference by DiS-C_3-(3) while transporting substrates across BBMV and OMMV.

In this study, the potential application of DiS-C_3-(3) dye as a biosensor for the membrane potential difference in BBMV and OMMV was validated using the following experiments: (1) investigating the addition of the extra-vesicular medium (pH 6.6, without substrate), which resulted in an instantaneous decrease in fluorescence intensity followed by a steady plateau (Figure 2). This initial change in intensity is likely due to the perturbation of the sample by the addition of the medium (Ries et al 1994), as when larger volumes were added a larger negative deflection was observed (results not shown). The following plateau indicates that there were no changes in the membrane potential and hence that no transport happened across the membrane for the control samples (Figure 2). (2) investigating the addition of the extra-vesicular medium (pH 6.6) containing Gly-Gln to the same vesicles, which resulted in the same decrease as the control ones, but followed by an increase in the fluorescence signal which then reached plateau. This increase in the fluorescence intensity indicated

depolarisation of the membrane potential, which is most likely due to the translocation of the substrates with H^+ across the membrane (Figure 2) (Ries et al 1994).

Chen et al. (1999) demonstrated that although PEPT1 and PEPT2 showed high sequence homology (approx. 50%), there were considerable differences in terms of substrates affinity, stoichiometry, and effects of depolarization and hyperpolarization. They demonstrated that PEPT2 can be inactivated by either increasing the pH or by hyperpolarization. Another study conducted by Daniel et al. (1991) used similar vesicle preparations isolated from the brush border membrane of the kidney and investigated the transport of just two concentrations of Gly-Gln 0.1 μM and 1mM as a function of pH gradients. They showed that Gly-Gln transport depends on the presence of a transmembrane pH gradient, although the high affinity transporter PEPT2 was significantly more sensitive to pH than the low affinity transporter PEPT1. In this study, BBMV and OMMV respresent S1 and S3 of the proximal tubule respectively (where PEPT1 and PEPT2 are respectively located), and this heterogeneous distribution of these transporters was confirmed by Western blot (Figure 1) (Shen et al 1999). At pH 6.6, both BBMV and OMMV demonstrated a change in membrane potential difference, which is likely to be due to the transport of the substrate across the membrane. The physiological pH level in S3 was measured to be 6.7-6.8 ((Vieira and Malnic 1968), where NHE3 is highly active to acidify the lumen (Daniel and Kottra 2004), so the nature of these transporters requires such environment. This can also give valuable insights regarding the differences between two important regions of the kidney in terms of small peptide handling and pharmokinetics at different pH.

In our experiments, different concentrations of small peptides containing 2-3 amino acids and selected peptidomimetic drugs were delivered to BBMV and OMMV pre-equilibrated with the extra-vesicular buffer and DiS-C_3-(3) dye at pH 6.6. This aimed to investigate the kinetic transport parameters (K_m and V_{max}) in two important regions of the renal proximal tubule (S1 and S2) using DiS-C_3-(3) dye. When the buffer (pH 6.6) containing the substrates was added to the vesicles (BBMV or OMMV) pre-equilibrated with DiS-C_3-(3) dye and the signal corrected for the addition of buffer (control), the fluorescence intensity increased indicating depolarisation change of the membrane potential, which is therefore most likely due to the translocation of the substrates with H^+ across the membrane (Figures from 3 to 5).

PEPT1 and PEPT2 cotransporters are heterogeneously distributed in rat kidney, suggesting a sequential process in the flux of peptides and peptide-like drugs in the proximal tubule of the nephron, initially by a low affinity / high capacity co-transporter, followed by a high affinity / low capacity co-transporter respectively. Since PEPT2 was found in larger abundance than PEPT1 in kidney (Leibach and Ganapathy 1996; Smith et al 1998), it was expected that peptides and peptidomimetic drugs are predominately transported by PEPT2 (Shen et al 1999). This assumption is supported by PEPT2 knockout mouse studies that showed PEPT2 protein is responsible for transporting about 85% of dipeptides (Daniel and Rubio-Aliaga 2003; Ocheltree et al 2005) and about 95% of cefadroxil (Shen et al 2007). In most cases, PEPT2 demonstrates higher affinity than PEPT1 in transporting peptidomimetic drugs such as β-lactam antibiotics, which contain an α-amino group and *p*-hydroxyphenyl group in their chemical configuration, at therapeutic concentrations (Inui et al 2000; Takahashi et al 1998). This is in agreement with our findings that OMMV (where PEPT2 is located) has a higher affinity for most substrates compared to BBMV (PEPT1), with cefadroxil having the highest affinity (table 1). This value is comparable and in a good correlation with previous studies

reporting that cefadroxil had a very high affinity for PEPT2 in the transfected cell line LLC-PK1 (Terada et al 1997) and to the native PEPT2 in BBMV isolated from rat kidneys (Inui et al 2000; Ries et al 1994; Takahashi et al 1998) where the transported K_m and K_i was around 3 μM. Valacyclovir had the second highest affinity in OMMV (table 1), and to compare it with cefadroxil, the former contains α-amino group and *p*-hydroxyphenyl while the latter contains just an α-amino group. In contrast, δ-amino-levulinic acid, which does not contain these groups showed the lowest affinity in OMMV (Table 1). These results are in good agreement with the literature (Bransch et al 2008), confirming that these functional groups in the chemical structure can play an important role in the recognition of substrates by PEPT2. Also, it suggests that S3 is the predominant region for transporting these substrates at low therapeutic concentration, while competing with the hydrolysed small peptides in the kidney.

On the other hand, in OMMV the transport of Gly-His (a positively charged dipeptide) demonstrated significantly higher afinnity than Gly-Gln (a neutral dipeptide) and Gly-Gly-His (a tripeptide), but showed significantly higher capacity for Gly-Gly-His (table 1). Although, the transport of Gly-Gln by OMMV was significantly higher in affinity and capacity than BBMV, no significant changes in affinity or capacity were observed between BBMV and OMMV for Gly-His (table 1). This could be explained by the charge-to-peptide stoichiometry of PEPT1 for acidic and neutral peptides which is 1:1 and its preference for positively charged dipeptides (Bransch et al 2008; Steel et al 1997), while the proton-to-substrate stoichiometry of PEPT2 for neutral peptides is 2:1 (Chen et al 1999). The extracellular pH plays a substantial role in increasing the affinity of both anionic and cationic compounds, such that decreasing the extracellular pH increases the affinity of anionic and neutral compounds, whereas increasing extracellular pH decreases the affinity of cationic ones (Amasheh et al 1997). It is clear that changing the pH and peptide charge can play a

substantial role in determining the transport by PEPT1 and PEPT2. Therefore, one explanation of the higher transport affinity and capacity of Gly-Gln (neutral dipeptide) by OMMV (table 1) is that the proton-to-substrate stoichiometry is 2:1, but also, that the positively charged dipeptide can increase the affinity and capacity of these transporters at physiological pH.

In this study, we have measured the transport kinetics of neutral and positively charged small peptides and selected peptidomimetic drugs delivered to BBMV and OMMV by using the membrane potential difference sensitive dye DiS-C_3-(3). This is the first report to infer the relative contribution of the S1 and S3 segments from the renal proximal tubule (which are represented by BBMV and OMMV containing PEPT1 and PEPT2 cotransporters respectively) to the tubular reabsorption of these substrates in the kidney. These findings could provide valuable insights regarding the transport efficiency between these different substrates and between PEPT1 and PEPT2 in handling nutrients and peptide-like drugs. This could be of importance when considering substrate or drug distribution in the body.

Conflict of interest

Apart from the funding sources (listed on the title page), the authors declare that they have no other conflicts of interest.

References

1. Alghamdi OA, King N (2012) Novel application of the dye, DiS-C_3-(3) as a biosensor of the renal membrane potential difference. International Journal of Bioscience, Biochemistry and Bioinformatics, 9 (4): 282-286.

2. Amasheh S, Wenzel U, Boll M, Dorn D, Weber WM, Clauss W, Daniel H (1997) Transport of charged dipeptides by the intestinal H+/peptide symporter PepT1 expressed in Xenopus laevis oocytes. Journal of Membrane Biology 155 (3):247-256

3. Anderson CM, Thwaites DT (2010) Hijacking solute carriers for proton-coupled drug transport. Physiology (Bethesda, Md) 25 (6):364-377. doi:10.1152/physiol.00027.2010

4. Beck JC, Sacktor B (1978) Membrane potential-sensitive fluorescence changes during Na+-dependent D-glucose transport in renal brush border membrane vesicles. Journal of Biological Chemistry 253 (20):7158

5. Biber J, Stieger B, Haase W, Murer H (1981) A high yield preparation for rat kidney brush border membranes. Different behaviour of lysosomal markers. Biochimica et Biophysica Acta 647 (2):169-176

6. Blostein R (1999) Structure-function studies of the sodium pump. Biochemistry and Cell Biology 77 (1):1-10. doi:10.1139/o99-018

7. Brandsch M, Knutter I, Bosse-Doenecke E (2008) Pharmaceutical and pharmacological importance of peptide transporters. The Journal of pharmacy and pharmacology, 60 (5):543-585. doi:10.1211/jpp.60.5.0002

8. Brown CD, King N, Simmons NL (1993) Co-expression of an anion conductance pathway with Na(+)-glucose cotransport in rat renal brush-border membrane vesicles. Pflugers Archiv European journal of physiology 423 (5-6):406-410

9. Cassano G, Maffia M, Vilella, Storelli C (1988) Effects of Membrane Potential on Na Cotransporters in Eel Intestinal Brush-Border Membrane Vesicles: Studies with Fluorescent Dye. The Journal of membrane biology 101:225-236

10. Chen X, Z., Zhu T, Smith D, E., Hediger M, A. (1999) Stoichiometry and kinetics of the high-affinity H+-coupled peptide transporter PepT2. Journalof biological chemistry, 274 (5):2773-2779. doi:10.1074/jbc.274.5.2773

11. Daniel H, Kottra G (2004) The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Archiv : European Jornal of Physiology 447 (5):610-618. doi:10.1007/s00424-003-1101-4

12. Daniel H, Morse EL, Adibi SA (1991) The high and low affinity transport systems for dipeptides in kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. The Journal of Biological Chemistry, 266 (30):19917-19924

13. Daniel H, Rubio-Aliaga I (2003) An update on renal peptide transporters. American Journal of Physiology Renal Physiology, 284 (5):F885-892. doi:10.1152/ajprenal.00123.2002

14. Donowitz M, Emmer E, McCullen J, Reinlib L, Cohen ME, Rood RP, Madara J, Sharp GW, Murer H, Malmstrom K (1987) Freeze-thaw and high-voltage discharge allow macromolecule uptake into ileal brush-border vesicles. American Journal of Physiology 252 (6 Pt 1):G723-735

15. Faller LD (2008) Mechanistic studies of sodium pump. Archives of Biochemistry and Biophysics, 476 (1):12-21. doi:10.1016/j.abb.2008.05.017

16. Inui K, Terada T, Masuda S, Saito H (2000) Physiological and pharmacological implications of peptide transporters, PEPT1 and PEPT2. Nephrology Dialysis Transplantation 15 Suppl 6:11-13

17. Jorgensen P (1986) Structrue, function and regulation of Na, K-ATPase in the kidney. . Kidney International 29 (1):10-20

18. Kaur J, Bachhawat AK (2009) A modified Western blot protocol for enhanced sensitivity in the detection of a membrane protein. Analytical Biochemistry, 384 (2):348-349. doi:10.1016/j.ab.2008.10.005

19. Kottra G, Spanier B, Verri T, Daniel H (2013) Peptide transporter isoforms are discriminated by the fluorophore-conjugated dipeptides beta-Ala- and d-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid. Physiological Reports, 1 (7):e00165. doi:10.1002/phy2.165

20. Kragh-Hansen U, Jorgensen KE, Sheikh MI (1982) The use of potential-sensitive cyanine dye for studying ion-dependent electrogenic renal transport of organic solutes. Spectrophotometric measurements. The Biochemical Journal, 208 (2):359-368

21. Kragh-Hansen U, Sheikh MI (1984) Serine uptake by luminal and basolateral membrane vesicles from rabbit kidney. The Journal of Physiology 354:55-67

22. Leibach FH, Ganapathy V (1996) Peptide transporters in the intestine and the kidney. Annual Review of Nutrition 16:99-119. doi:10.1146/annurev.nu.16.070196.000531

23. Ocheltree SM, Shen H, Hu Y, Keep RF, Smith DE (2005) Role and relevance of peptide transporter 2 (PEPT2) in the kidney and choroid plexus: in vivo studies with glycylsarcosine in wild-type and PEPT2 knockout mice. The Journal of Pharmacology and Experimental Therapeutics, 315 (1):240-247. doi:10.1124/jpet.105.089359

24. Plášek J, Dale RE, Sigler K, Laskay G (1994) Transmembrane potentials in cells: a diS-C_3(3) assay for relative potentials as an indicator of real changes. Biochimica et Biophysica Acta, 1196: 181-190

25. Ries M, Wenzel U, Daniel H (1994) Transport of cefadroxil in rat kidney brush-border membranes is mediated by two electrogenic H+-coupled systems. The Journal of Pharmacology and Experimental Therapeutics, 271 (3):1327-1333

26. Rubio-Aliaga I, Daniel H (2008) Peptide transporters and their roles in physiological processes and drug disposition. Xenobiotica; the fate of foreign compounds in biological systems 38 (7-8):1022-1042. doi:10.1080/00498250701875254

27. Shen H, Ocheltree SM, Hu Y, Keep RF, Smith DE (2007) Impact of genetic knockout of PEPT2 on cefadroxil pharmacokinetics, renal tubular reabsorption, and brain penetration in mice. Drug Metabolism and Disposition 35 (7):1209-1216. doi:10.1124/dmd.107.015263

28. Shen H, Smith DE, Yang T, Huang YG, Schnermann JB, Brosius III FC (1999) Localization of PEPT1 and PEPT2 proton-coupled oligopeptide transporter mRNA and protein in rat kidney. American Journal of Physiology-Renal Physiology 276 (5):F658-F665

29. Smith DE, Pavlova A, Berger UV, Hediger MA, Yang T, Huang YG, Schnermann JB (1998) Tubular localization and tissue distribution of peptide transporters in rat kidney. Pharmaceutical Research 15 (8):1244-1249

30. Steel A, Nussberger S, Romero M, Boron W, Boyd C, Hediger M (1997) Stoichiometry and pH dependence of the rabbit proton-dependent oligopeptide transporter PepT1. The Journal of Physiology 498 (Pt 3):563

31. Takahashi K, Nakamura N, Terada T, Okano T, Futami T, Saito H, Inui KI (1998) Interaction of beta-lactam antibiotics with H+/peptide cotransporters in rat renal brush-border membranes. The Journal of Pharmacology and Experimental Therapeutics, 286 (2):1037-1042

32. Terada T, Saito H, Mukai M, Inui K (1997) Recognition of beta-lactam antibiotics by rat peptide transporters, PEPT1 and PEPT2, in LLC-PK1 cells. The American Journal of Physiology, 273 (5 Pt 2):F706-711

33. Vayro S, Simmons NL (1996) An effect of Ca2+ on the Intrinsic Cl(-)-conductance of rat kidney cortex brush border membrane vesicles. The Journal of Membrane Biology 150 (2):163-173

34. Vieira F, Malnic G (1968) Hydrogen ion secretion by rat renal cortical tubules as studied by an antimony microelectrode. American Journal of Physiology--Legacy Content 214 (4):710-718

35. Wright SH, Krasne S, Kippen I, Wright EM (1981) Na+-dependent transport of tricarboxylic acid cycle intermediates by renal brush border membranes. Effects on fluorescence of a potential-sensitive cyanine dye. Biochimica et Biophysica Acta 640 (3):767-778

Figure and Table Legends

Fig. 1 The presence of PEPT1 and PEPT2 in BBMV and OMMV, respectively, isolated from the kidneys of adult rats. The protein concentration loaded in each gel was constant 45µg. As labelled in the graph PEPT1 was present in BBMV, and PEPT2 was present in OMMV.

Fig. 2 Changes in fluorescence intensity measured upon Gly-Gln (1000µM) addition to either BBMV or OMMV. Controls (no substrate addition) for these samples were run as indicated in this graph. The arrow indicates the point at which the Gly-Gln was added. Data shown are taken from a single experiment representative of 7 such experiments.

Fig. 3 Change in fluorescence (ΔF/0.02s, > 0) as a function of substrate concentration delivered to the BBMV at pH 6.6. Squares joined with a continuous line represent the values obtained for Gly-His. Circles joined with a dashed line represent the values obtained for Gly-Gln. Triangles joined by a bold dashed line represent the values for Cefadroxil. Curves were fitted according to the Michaelis-Menten equation. Data shown are means ± SE, where n=8.

Fig. 4 Change in fluorescence (ΔF/0.02s, > 0) as a function of substrate concentration delivered to the OMMV at pH 6.6. Squares joined with a continuous line represent the values obtained for Gly-His. Circles joined with a dashed line represent the values obtained for Gly-Gln. Triangles joined with a bold dashed line represent the values for Gly-Gly-His. Curves were fitted according to the Michaelis-Menten equation. Data shown are means ± SE, where n=8

Fig. 5 Change in fluorescence (ΔF/0.02s, > 0) as a function of substrate concentration delivered to the OMMV at pH 6.6. Squares joined with a continuous line represent the values obtained for Valacyclovir. Circles joined with a dashed line represent the values obtained for Cefadroxil. Triangles joined with a bold dashed line represent the values for δ-amino-levulinic acid. Curves were fitted according to the Michaelis-Menten equation. Data shown are means ± SE, where n=6.

Table 1. The Km (μM) and Vmax (ΔF/s/mg protein) kinetic parameters for different substrates in two regions of the kidney (BBMV and OMMV). The affinity and velocity values of these substrates are shown in order from the highest to the lowest. Data shown are means ± SE, where n=6-8.