2018-05

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Alghamdi, OA

10.1016/j.bbamem.2017.12.021
Biochimica et Biophysica Acta - Biomembranes
Elsevier

A new use of β-Ala-Lys (AMCA) as a transport reporter for PEPT1 and PEPT2 in renal brush border and outer medulla membrane vesicles.

[1,3]Othman A. Alghamdi, [2]Nicola King, [1]Graham L. Jones, [1]Pierre D.J. Moens

[1]School of Science and Technology, University of New England Armidale, NSW 2351, Australia

[2]School of Biomedical and healthcare Sciences, Peninsula School of Medicine and Dentistry, Plymouth University, Plymouth PL4 8AA, UK

[3]Faculty of Science, Department of Biochemistry, Centre for Science and Medical Research, University of Jeddah, KSA

Correspondence to: Othman Alghamdi

Faculty of Science, Department of Biochemistry, Centre for Science and Medical Research, University of Jeddah, KSA

Email: biomedoth@gmail.com

Tel.: +966127451739

ORCID.org/0000-0001-5673-284X

Abstract

Integral membrane proteins PEPT1 and PEPT2 are essential for reabsorbing almost all hydrolysed or filtered di- and tripeptides alongside a wide range of peptidomimetic drugs in the kidney. The aim of this study was to investigate the potential use of the fluorophore-conjugated dipeptide β-Ala-Lys (AMCA) as a biosensor for measuring peptide transport activity in brush border (BBMV) and outer medulla (OMMV) membrane vesicles (which represent PEPT1 and PEPT2 respectively). BBMV and OMMV were isolated using a dual magnesium precipitation and centrifugation technique. Intravesicular fluorescence accumulation was measured after incubating extra-vesicular media at pH 6.6 and different concentrations of β-Ala-Lys (AMCA) with vesicles pre-equilibrated at pH 7.4. Both BBMV and OMMV showed accumulation of an intravesicular fluorescence signal after 20 min incubation. Changing the extra-vesicular pH to 7.4 caused a significant reduction in the β-Ala-Lys (AMCA) uptake into BBMV at concentrations > 100 μM. When different concentrations of dipeptide, Gly-Gln was added, there was a significant inhibition of 100 μM β-Ala-Lys (AMCA) uptake into BBMV and OMMV, reaching 69 % and 80 %, respectively. Kinetic analysis of β-Ala-Lys (AMCA) at 20 min showed that the K_m and V_{max} were 783.7 ± 115.7μM and 2191.2 ± 133.9 ΔF /min/mg for BBMV, while OMMV showed significantly higher affinity, but lower capacity at K_m = 93.6 ± 21.9μM and V_{max} = 935.8 ± 50.2 ΔF /min/mg. These findings demonstrate the applicability of β-Ala-Lys (AMCA) as a biosensor to measure the transport activity of the renal-type PEPT1 and PEPT2 in BBMV and OMMV respectively.

Key Words: β-Ala-Lys (AMCA); Gly-Gln; BBMV; OMMV; PEPT co-transporters.

Introduction

Di- and tripeptide transporters PEPT1 and PEPT2 located in the renal proximal tubule play substantial roles in the body protein balance by reabsorbing any enzymatically hydrolysed or filtered oligopeptides. Equally important, is the transport of peptide-like drugs such as β-lactam antibiotics, some ACE inhibitors, and the anti-tumour drugs, Bestatin, and δ-amino-levulinic acid and selected antiviral prodrugs [15,2]. The localisations of PEPT1 and PEPT2 cotransporters in the kidney are respectively restricted to segments 1 and 3 of the renal proximal tubule. The former is a low-affinity/high-capacity cotransporter, while the latter is a high-affinity/low-capacity cotransporter [18].

Several studies have used the fluorophore-conjugated dipeptide, β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-Ala-Lys (AMCA)) and D-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid (D-AL-AMCA) to visualise and quantify transport by the PEPT cotransporters. Examples of these studies include the application of D-AL-AMCA as a fluorescent reporter for transport in: LLC-PK1cells [21], mouse intestine [7], renal proximal tubule in rat [8], and in PEPT2-deficient mice [16]. In addition, β-Ala-Lys (AMCA) was first synthesised and used as a fluorescent reporter tool by Dieck et al. [5] for studying the uptake of dipeptides and peptidommetic drugs in astrocytes that express the PEPT2 cotransporter. Subsequent studies revealed the potential application of this reporter as a tool for characterising oligopeptide transport in: the mammalian enteric nervous system, intestinal-like organoids in human pluripotent stem cells, S. cerevisiae expressing Ptr2p, *E. coli* expressing *ydgR* and in rat thyroid follicular cells [17,19,14,11,20]. However, a study conducted by Kottra et al. [12] reported that D-AL-AMCA was not a substrate in the intestine when they compared wild type mice with PEPT2-deficient mice. The same study demonstrated a systematic comparison

between the two fluorescently labelled dipeptides using *Xenopus* oocytes that expressed a wide range of species-derived PEPT1 and PEPT2 isoforms. It was concluded that although β-Ala-Lys (AMCA) was not a good tool for investigating peptide transport in *Xenopus* oocytes expressing different isoforms of PEPT1, it was a good substrate for the renal-type PEPT cotransporters. In accordance with the discussion above, we report for the first time the application of β-Ala-Lys (AMCA) as a tool for investigating oligopeptide transport in BBMV and OMMV isolated from rat kidneys.

Materials and Methods

Chemicals

The fluorophore-conjugated dipeptide, β-Ala-Lys (AMCA) (Figure 1) was purchased from Bio Trend Chemicals (Destin, USA). All the other amino acids, chemicals and reagents were of analytical quality and acquired from SIGMA (Australia), Chem-Supply and AppliChem.

Kidneys

Male Wistar rats (6-7 months old) were obtained from the breeding colony maintained by the Animal House at the University of New England (UNE). The rats were sacrificed by stunning and cervical dislocation and their kidneys were snap frozen in liquid nitrogen prior to placing them at -80 °C. 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).

Preparation of BBMV and OMMV

Thin slices of superficial cortex (~1 mm thick) and outer medulla (characterised by red stripes) were dissected using forceps and a scalpel. These slices were then used to isolate BBMV and OMMV respectively by a dual-stage Mg^{++} /EGTA precipitation method [1,3,4]. After purifying the vesicles, they were suspended in intravesicular medium containing (in mM): 100 mannitol, 100 KH_2PO_4 and 10 4-(2-hydroxymethyl)-1-piperazineethanesulonic acid (HEPES) (pH 7.4 with tris(hydroxymethyl)aminomethane (TRIS)) and centrifuged at 31,000×g for 20 min at 4 °C. The supernatant was discarded, and the pellets were re-suspended in 500µl of the same solution and then pre-equilibrated by freeze-thaw fractionation [6]. The quality of the vesicles was examined by measuring the specific activities and enrichments of two marker enzymes, alkaline phosphatase and leucine aminopeptidase. All of the enrichments were >10 fold indicating uncontaminated and good vesicles. Bradford's method was used to quantify protein concentration [3]. The expression of PEPT1 and PEPT2 proteins in BBMV and OMMV respectively was confirmed by Western blot (results not shown).

Optimisation of β-Ala-Lys (AMCA) uptake as a function of time

Previous studies using β-Ala-Lys (AMCA) as a transport reporter in brain cells [5], enteric nervous tissue [17], and oocytes expressing various isoforms of oligopeptide cotransporters [12] reported optimal incubation periods of 2-3 hours. However, other studies using D-Ala-Lys (AMCA) in the kidney in vivo or in tubule segments were able to detect staining after 5-30 mins [8,16]. In our experiments, transport was measured using a different kidney preparation, namely BBMV and OMMV, hence it was required to optimise the incubation period. These experiments revealed that the best incubation periods to use were 20 minutes to 3 hours.

The fluorophore-conjugated dipeptide, β-Ala-Lys (AMCA) was prepared to its maximum soluble concentration 1 mg/ml (2.32mM, according to Bio Trend) in extravesicular buffer containing (in mM): 100 KH_2PO_4, 100 mannitol, and 10 2-(N-Morpholino) ethanesulfonic acid (MES)-Tris (pH 6.6). The experiment was started by mixing vesicles (containing 120 or 45µg proteins) with an appropriate volume of the incubating buffer and with different concentrations of β-Ala-Lys (AMCA) (5-2000µM) to a total volume of 250µl in Eppendorf tubes. These tubes were wrapped with aluminium foil and left at 20 °C for different periods, ranging from 20 minutes to 3 hours. After that, the transport was stopped by adding the mixture into centrifuge tubes containing 5ml of ice-cold extravesicular buffer prior to spinning at 31,000×g for 20 minutes at 4°C. The supernatant was discarded, and the pellet was washed in the centrifuge tubes 3 times, each with 3ml of the ice-cold extravesicular buffer. This was followed by re-suspending the pellet in 500µl of the extravesicular buffer using 21 x 1/5 gauge needle attached to a 1ml syringe. Suspensions of different concentrations incubated for different periods were transferred into 96-well black microplates in triplicate by pipetting 165µl in each well. Furthermore, 500µl from each wash 1-4 was also checked to confirm the elimination of nonspecific residual substrates. A microplate fluorimeter was used to measure fluorescence emission from 400-700nm with 5nm steps at an excitation wavelength of 350nm, and the maximal emission reading at 455nm was chosen as reported previously [5].

Substrate competition and pH-dependence studies

The competitive transport experiments were performed by adding an appropriate amount of extravesicular buffer containing 100µM β-Ala-Lys (AMCA) and 1.5-10mM Gly-Gln as a

positive control or 10mM of single amino acids as negative controls (Gln, His, and N-acetylcysteine). After mixing these components, either BBMV or OMMV containing 45 µg of proteins were added by pipetting up and down. In addition, the effect of pH on the transport of β-Ala-Lys (AMCA) by BBMV was examined for further confirmation of the proton-dependent characteristic of PEPT cotransporters. BBMV containing 45µg of proteins were mixed with an appropriate volume of either extravesicular (pH 6.6) or intravesicular (pH 7.4) buffer and with different concentrations of β-Ala-Lys (AMCA) 5-2000µM to a final volume of 250µl in Eppendorf tubes. All samples (competitive experiments and pH experiments) were kept in the dark for 20 minutes at 20 °C prior to proceeding to the fluorescence measurements as explained above.

Statistics and presentation

All values are presented as means ± SEM for n= 3-7 (in triplicates) independent measurements. Student's t-test was then calculated for paired or unpaired samples as appropriate and considered significantly different when $P<0.05$. Appropriate data were fitted to the Michaelis-Menten curve using the equation ($V = \frac{Vmax\,[S]}{Km+[S]}$) from which V_{max}, K_m, and R^2 values were calculated using ORIGIN41 software (OriginLap Corporation). The curve fit obtained displayed R^2 values >90. V_{max} values were calculated as ΔF/20 min/45µg which were converted to ΔF/min/mg. K_m values are presented as µM

Results

Optimisation for time course of β-Ala-Lys (AMCA) uptake

Different incubation time (20 minute-3 hours) of 500μM β-Ala-Lys (AMCA) with BBMV and OMMV showed a corresponding increase of the fluorescence intensity (Figure 2). This increase is not due to the presence of labelled dipeptide in the extravesicular medium since after wash 3 and 4 no fluorescence can be detected in the medium (Figure 3). 20 minute incubation time and 3 washing were chosen as the conditions for this experiment.

Kinetic measurements of β-Ala-Lys (AMCA) uptake

The kinetic parameters for the uptake of β-Ala-Lys (AMCA) under various concentrations ranging from 5-2000μM (the maximum concentration tested was limited by the solubility of β-Ala-Lys (AMCA)) into these vesicles (45μg protein) was calculated by fitting the data using Michaelis-Menten equation (Figure 4). It was clear that BBMV (circles, solid line) were capable of transporting this substrate with low affinity K_m= 783.7 ± 115.7μM and high capacity V_{max} = 2191.2 ± 133.9 ΔF/min/mg. In comparison, OMMV (triangles, dashed line) revealed significantly higher transport affinity K_m= 93.6 ± 21.9μM than that of BBMV (P= 0.002). However, OMMV showed significantly lower capacity V_{max} = 935.8 ± 50.2 ΔF/min/mg than that of BBMV in transporting β-Ala-Lys (AMCA) (P= 0.001).

Substrate specificity and competition experiments

In the first place, the specificity of β-Ala-Lys (AMCA) was investigated using Gly-Gln, which is a well-characterised substrate for both PEPT1 and PEPT2 [12,4]. Figure 5 shows the effect that different concentrations of Gly-Gln (1.5, 2, 4 and10 mM) had on the uptake of 100μM of β-Ala-Lys (AMCA) into BBMV (squares, solid line) and OMMV (circles, dashed line). Irrespective of the vesicle preparation investigated, there was a decrease in the percentage of fluorescence intensity as the concentration of Gly-Gln increased. However, at all concentrations of Gly-Gln, the percentage of fluorescence intensity was significantly lower in the OMMV compared to the BBMV (**P= 0.04, 0.001, 0.0002, 0.0001 respectively). In contrast, the addition of 10mM of the single amino acids Gln and acetylcysteine not transported by PEPT1 or PEPT2 and L-His (substrate of PHT1 and PHT2 [14]) did not affect β-Ala-Lys (AMCA) uptake compared to control (no added amino acids) (Figure 6)

PH- dependence of β-Ala-Lys (AMCA) uptake

BBMV were incubated with different concentrations of β-Ala-Lys (AMCA) at pH 6.6 (solid line) and 7.4 (dashed line) (Figure 7). At both pHs, the ΔF slightly and non-significantly increased from 5-100μM. Thereafter, the ΔF linearly increased at pH 6.6 and was for all concentrations significantly greater than that measured at pH 7.4.

Discussion

In the last two decades, fluorophore-conjugated dipeptides such as β-Ala-Lys (AMCA) and D-AL-AMCA have been used as hydrolysis-resistant reporters. Both of these biosensors have been utilised to visualise and measure the activity of PEPT isoforms located in different cells and tissues [14,5,21,17,7,9,10]. Until now, two studies have demonstrated the potential application of D-AL-AMCA as a fluorescently labelled substrate for oligopeptide transporters in the kidney. One showed that administration of 25μM D-AL-AMCA led to fluorescence accumulation after 30 min in the proximal tubule outer and inner cortex and outer medulla, suggesting that PEPT1 and PEPT2 were mediating D-AL-AMCA uptake [8]. In the second study, Rubio-Aliaga et al. [16] reported that although fluorescence accumulation was seen in the cortex and outer medulla of wild-type mice after 5 min, *PEPT2* knockout mice revealed no fluorescence signal in the outer medulla with only low fluorescence signal in the outer cortex, known as the region of PEPT1 cotransporter. On the other hand, only one study has reported the possible application of β-Ala-Lys (AMCA) as a peptide substrate for the rat renal-type PEPT1 and PEPT2 isoforms expressed in *Xenopus* oocytes [12]. Based on these data, we developed a new method to investigate the potential use of β-Ala-Lys (AMCA) as a substrate for the native renal-type PEPT1 and PEPT2 cotransporters using BBMV and OMMV isolated form rat kidneys.

Different incubation times (20 minute-3 hours) of 500 μM β-Ala-Lys (AMCA) regardless of the type of vesicles (BBMV and OMMV) resulted in an increase in fluorescence (Figure 2), suggesting that these vesicles were able to translocate β-Ala-Lys (AMCA) as efficiently in 20 minutes as the other incubation times. β-Ala-Lys (AMCA) was also a good fluorophore-conjugated substrate as it did not quench during the time of the experiments. The absence of

differences in the transport of β-Ala-Lys (AMCA) between the 4 incubation times could indicate that the transport equilibrium would have been reached before 20 minute. This would be supported by another study [13] which suggested that the transport of substrates could occur in milli-to-microsecond time scale. Also, other studies conducted by Rubio-Aliaga et al. [16],Groneberg et al. [8] detected and visualised the fluorescence of D-AL-AMCA in the kidney cortex and outer medulla 5 min after the injection. The study of *Xenopus* oocytes expressing various PEPT isoforms reported that washing oocytes 3 times was enough to eliminate extra-oocytes or un-transported substrates [12]. Likewise, washing the pellets of BBMV and OMMV 3 times with extra-vesicular buffer was sufficient to completely remove remaining extra-vesicular β-Ala-Lys (AMCA) (Figure 4).

Together with the increased intensity observed in BBMV and OMMV as the substrate concentration increases, the experiments in Figure 5, demonstrate the competition between β-Ala-Lys (AMCA) and Gly-Gln, a well-known substrate for PEPT1 and PEPT2 cotransporters [17,8,12], by showing a reduction of fluorescence accumulation to about 32 % and 20 % in BBMV and OMMV, respectively Figure 5. In addition, the competition experiments using single amino acids (Figure 6) such as Gln and N-acetylcysteine did not inhibit β-Ala-Lys (AMCA) uptake, nor did L-histidine, which is a substrate for PHT1 and PHT2 [17,14]. These findings strongly support that the fluorescence accumulation inside the vesicles corresponds to the uptake of this substrate by the PEPT cotransporters. They also confirm that the renal-type PEPT cotransporters mediate the uptake of β-Ala-Lys (AMCA), with higher affinity in OMMV [8,16,12].

The effect of pH gradients on PEPT cotransporters located in BBMV was studied by Daniel et al. [4] who reported that the low affinity transporter (PEPT1) can function in the absence of a pH gradient, but with low transport capacity. They studied the transport of just two concentrations of Gly-Gln (0.1 μM and 1mM) as a function of pH gradients. These results are in agreement with this study. Indeed, when different concentrations of β-Ala-Lys (AMCA) were incubated with BBMV (which is known as the site of PEPT1 cotransporter [18]), there was a small uptake of the substrate (illustrated by the slight increase in intensity) in the absence of pH gradients, but with low capacity (Figure 7).

Michaelis-Menten analysis of β-Ala-Lys (AMCA) uptake as a function of concentrations indicated that OMMV mediated the transport of β-Ala-Lys (AMCA) with significantly higher affinity, but lower capacity than BBMV. This could reflect the properties of PEPT1 and PEPT2 cotransporters, which are respectively expressed in these vesicles. A *PEPT2* knockout study reported that no fluorescence signal was detected in the outer medulla when 100 μM of D-AL-AMCA was injected into the mice, but very small signals were seen in the outer cortex [16]. Very weak fluorescence in the outer cortex region, where PEPT1 is expressed, could result from low affinity for this substrate due to the bulky and hydrophobic-side chain modifications, while PEPT2 seems to better tolerate the bulkiness of the fluorophore [12]. Another possibility is that the low intensity of fluorescence reported by Rubio-Aliaga et al. [16],Groneberg et al. [8] is due to the injection of a small concentration. However, in our study it is clearly shown that both BBMV and OMMV representing the renal-type PEPT1 and PEPT2 cotransporters are both capable of transporting β-Ala-Lys (AMCA) with low-affinity/high-capacity and high-affinity/low-capacity respectively.

In conclusion, BBMV and OMMV isolated from two regions of the renal proximal tubule (superficial cortex and outer medulla), which represent the renal-type PEPT1 and PEPT2 cotransporters, mediate the uptake of β-Ala-Lys (AMCA). Therefore, it can be used as a tool to visualise and measure the activity of oligopeptide transport at least in the kidney.

Acknowledgments

This work was supported by the Saudi Ministry of Higher Education and University of New England. We also would like to thank Mr. Brian Cross and Mr. Jonathon Clay for their excellent technical assistance.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1 Chemical structure of the fluorophore-conjugated dipeptide β-Ala-Lys (AMCA).

Fig. 2 Increases of the fluorescence as a function of time for 500μM β-Ala-Lys (AMCA) incubated with 120μg protein of the BBMV (solid bars) and OMMV (downward diagonal lined bars) at pH 6.6. Incubation periods were 20 minute, 1 hour, 2 hours, and 3 hours. Data shown are means ± SE, where n= 3 (in triplicate).

Fig. 3 Decreases in fluorescence as a function of number of washes for BBMV (dashed line) and OMMV (solid line) incubated with 1mm β-Ala-Lys (AMCA). Each wash contained 3 ml

of the extra-vesicular buffer, of which 500µl was taken to measure the fluorescence. Data shown are means ± SE, where n= 3 (in triplicate).

Fig. 4 Increases in fluorescence as a function of β-Ala-Lys (AMCA) concentration incubated for 20 min with either BBMV (solid line) or OMMV (dashed line) containing 45µg proteins at pH 6.6. The curves are the fit of the Michaelis-Menten equation (r^2 >90). Data shown are means ± se, where n= 6 (in triplicate).

Fig. 5 Inhibition of the transport of 100µm β-Ala-Lys (AMCA) transport into BBMV (solid line) and OMMV (dashed line) by Gly-Gln (0-10mm) at pH 6.6. Data shown are means ± SE, where n= 6 (in triplicate). *p<0.05 and **p<0.01 compared to OMMV.

Fig. 6 Competitive inhibition of 100µm β-Ala-Lys (AMCA) transport into BBMV by single amino acids (10mm): glutamine (Gln) histidine (His) and N-acetylcysteine. Data shown are means ± SE, where n= 3 (in triplicate)

Fig. 7 Changes in fluorescence as a function of β-Ala-Lys (AMCA) concentration incubated with BBMV at pH 6.6 (solid line) and at pH 7.4 (dashed line). **p<0.001 compared to pH 6.6. Data shown are means ± SE, where n= 3-4 (in triplicate).