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U373-MG cells express PepT2 and accumulate the fluorescently tagged dipeptide-derivative β-Ala-Lys-N_ε-AMCA

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ABSTRACT

Aim of this study was to examine the dipeptide transport of β-Ala-Lys-N_ε-AMCA in the human glioma cell line U373-MG and its potential regulation by diverse hormones and culture media. A mixed glial primary cell culture of the newborn rat served as reference cell system. β-Ala-Lys-N_ε-AMCA (β-Ala-Lys-N_ε-7-amino-4-methyl-coumarin-3-acetic acid) is a highly specific reporter substrate to investigate the dipeptide transport system PepT2. We were able to demonstrate that U373-MG cells express PepT2-mRNA and translocate β-Ala-Lys-N_ε-AMCA via PepT2 into the cytoplasm. Previous results demonstrated that β-Ala-Lys-N_ε-AMCA specifically accumulates in differentiated and dedifferentiated astrocytes but neither in differentiated nor dedifferentiated oligodendrocytes and in neurons. U373-MG cells were incubated with estradiol, testosterone, thyronine, dexamethasone, dibutyryl cyclic adenosine monophosphate and tetradecanoylphorbol acetate in order to detect potential substance-dependent changes in dipeptide uptake. There was no significant increase or decrease of β-Ala-Lys-N_ε-AMCA-uptake after stimulation. Northern blot analyses confirmed that PepT2-mRNA is expressed in U373-MG and glial cells but showed no regulation of PepT2-mRNA expression in both cell types. Future investigations might offer the opportunity of an anti-tumor therapy with cytotoxic agents linked to a dipeptide-derivative such as β-Ala-Lys.

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Dipeptide transport systems have been cloned and characterized from the mammalian duodenum and kidney and were designated PepT1 and PepT2 [7,10,12,18,19,38]. PepT2 is mainly expressed in the kidney and also in many other tissues with 50% amino acid identity to PepT1 but a higher affinity for its substrates [29,37]. Two recent studies suggest that PepT2 might function as scavenger-system for peptide fragments resulting from neuropeptide metabolism [37,39]. In this context it is important to mention that PepT2 knock-out mice (−/−) were viable and without obvious physiological and histochemical abnormalities of the brain and kidney [28,32]. PepT1 and PepT2 are of pharmacological interest, because they transport diverse drugs like β-lactam antibiotics or ACE-inhibitors [3,11,13,15,17,27,33,34].

Little is known about hormonal influences on PepT2 expression. Previous studies described PepT2 being regulated by intracellular Ca^{2+} or epidermal growth factor [5,40]. Hypothyroidism [9] and thyroidectomy [22] of rats resulted in increased levels of renal PepT2 expression, suggesting that peptide homeostasis or drug pharmacokinetics might change during altered thyroid function. 5/6 nephrectomized rats exhibited a selectively up-regulated renal expression of PepT2 2 weeks after surgery [24,35]. Rat intestinal PepT1 expression showed a diurnal rhythm, but renal PepT2 expression did not [25]. These findings suggest that regulatory mechanisms concerning both transport systems might differ between tissues and isoforms.

Three independent groups demonstrated an uptake of β-Ala-His and glycyl-sarcosine in astroglia-rich cell cultures [1,30,33,39,41,42]. However, basic studies concerning dipeptide uptake in human astrocytic tumour cell lines are missing. U373-MG is an astrocytoma cell line used as an *in vitro* model of malignant glioma. β-Ala-Lys-N_ε-AMCA is a highly specific reporter molecule to investigate the human dipeptide transport system PepT2 on glial cells. It is a synthetic, hydrolysis- and peptidase-resistant dipeptide with high affinity to PepT2, containing a fluorescent reporter molecule to directly visualize and quantify its specific cellular accumulation.

The reagents were obtained from the following sources: AMCA-ONSu from Pierce (Rockford, IL); N_ε-Fmoc-Lys from Saxon Biochemicals (Hannover, Germany); Boc-β-Ala-ONSu from Bachem (Heidelberg, Germany); fetal calf serum (FCS) from GIBCO

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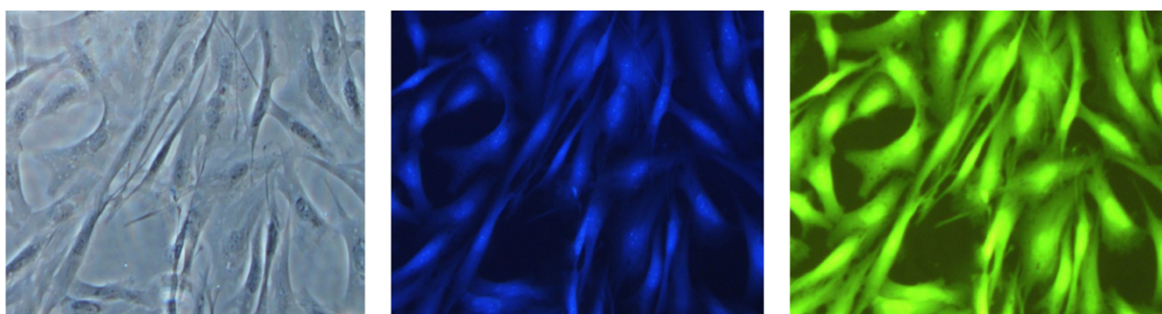

Fig. 1. U373-MG cells were incubated for 2 h with 24 μM β-Ala-Lys-N_ε-AMCA (37 °C) and afterwards stained against GFAP. The results were visualized by fluorescence microscopy. Left: phase contrast; middle: uptake of β-Ala-Lys-N_ε-AMCA by U373-MG cells; right: GFAP-labelling (magnification: 100×).

(Eggenstein, Germany); polyclonal cow anti-glial fibrillary acidic protein and the secondary antibody from Dianova (Hamburg, Germany); [^{32}P]-deoxy-CTP (5000 Ci/mmol) from Hartmann Analytic (Braunschweig, Germany), nylon membranes (Nytran NY 12N) from Schleicher & Schuell (Dassel, Germany). Magnetic oligo$(dT)_{25}$ polystyrene beads from Dynal (Hamburg, Germany); X-Omat-films, NTB-2 nuclear emulsion, and Rapid fix from Kodak (Integra Bioscience, Fernwald, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) and Sigma–Aldrich (Deisenhofen, Germany).

Synthesis, preparation and isolation of β-Ala-Lys-N_ε-AMCA was done by Prof. Dr. Karl Bauer, former Max-Planck-Institute for experimental Endocrinology, Hannover, Germany, using the N-hydroxysuccinimide ester method [2].

U373-MG cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, Biochrom, Berlin, Germany), supplemented with 10% FCS (Biochrom), penicillin G (100 U/ml, Biochrom) and streptomycin-sulfate (100 mg/ml, Biochrom). Cells were incubated under standard conditions (37 °C, 10% CO_2, 90% air, saturating and humidified atmosphere) and passaged by trypsinization (0.1% trypsin). The cells were used for experiments after 2 days of culture.

4–8 neonatal rats (Sprague–Dawley) were washed with 70% ethanol, sterile water and decapitated under sterile work bench conditions. The brains were removed and mechanically dissociated in DMEM using two different sterile nylon meshes (250 and 125 μm diameter). Afterwards, the cells underwent an enzymatical (0.1% trypsin) digestion for 30 s. The preparation was performed in a modified way as described earlier by Crang and Blakemore [6]. After seeding on 24-well plates (2×10^5 viable cells/well in 2 ml DMEM with 10% FCS and antibiotics), the cells were incubated under saturating humidity in an atmosphere of 10% CO_2 and 90% air. The cells were used for the experiments after 2 days of culture.

A mixed glial primary cell culture was established. After 15 days the number of astrocytes was about 90%. To obtain glial conditioned medium (GCM), DMEM was discarded and the cells were washed three times and subsequently cultured in serum-free medium. After 24 h the glial conditioned medium was collected and stored at −20 °C.

The following substances were used in final concentrations in the cell culture: estradiol (3 nM), testosterone (3 nM), 3,3,5-triiodo-L-thyronine (3 nM), dexamethasone (100 nM), dibutyryl cyclic adenosine monophosphate [31] (1 mM) and tetradecanoylphorbol acetate (100 mM). All chemicals were dissolved in DMEM and obtained from Merck (Darmstadt, Germany) or Sigma–Aldrich (Deisenhofen, Germany). Drug treatment was carried out during a 24 h incubation of the cells in serum-free medium.

The data were analysed using Prism version 5.02 for Windows (GraphPad Software, San Diego, USA). Student's *t*-test was performed for the comparison of the dipeptide uptake capacity of both U373-MG and glial cells.

Cell pellets (5×10^7 cells) from 75 cm^2 flasks were homogenized with a Teflon-glass homogenizer in 4 ml lysis buffer consisting of 100 mM Tris, 0.5 M LiCl, 10 mM EDTA, 1% sodium-lauryl-sulfate, and 5 mM dithiothreitol, pH 8.0. Polyadenylated poly$(A)^+$-enriched RNA was isolated using magnetic oligo$(dT)_{25}$ polystyrene beads (Dynal, Hamburg, Germany). Poly$(A)^+$-enriched RNA was size-separated in a denaturing agarose gel (2.2 M formaldehyde, 1.5% agarose), transferred to nylon membranes (Nytran NY), and cross-linked by ultraviolet irradiation. Hybridization was performed at 42 °C for 16 h in 50% formamide, 0.5% sodium-dodecyl-sulfate, 100 μg/ml salmon sperm DNA, and 6 SSPE (1 SSPE = 0.15 M NaCl, 15 mM sodium–phosphate, 2 mM EDTA, pH 7.4) with 100 ng of a 450-bp hPepT2 polymerase chain reaction (PCR)-cDNA fragment (housekeeping-gene: human cyclophilin A, 1-kb labelled PCR-cDNA, Clontech, Heidelberg, Germany), which was randomly labeled with (^{32}P)-deoxy-CTP to high specific activities (>10^9 cpm/μg) using a random-primer DNA labeling kit (Stratagene, Heidelberg, Germany). The membrane was washed to a final stringency of 0.2 SSPE, 0.3% SDS at 56 °C, and then exposed to X-ray film X-Omat for 7 days. The membrane was stripped by washing twice in 0.1 SSPE, 0.3% SDS at 99 °C for 10 min.

Recently, a dipeptide transport system on neonatal astrocytes turned out to be the human dipeptide transport system PepT2 [39]. Additionally, an equivalent dipeptide transport system exists in human primary cell cultures of glioblastomas and astrocytomas [43].

In our experiments β-Ala-Lys-N_ε-AMCA was found to be an excellent molecule for cytochemical uptake-studies in U373-MG cells (Fig. 1, left). After incubation with this substrate on day 2 of cell culture all tumour cells exhibited a bright blue fluorescence (Fig. 1, middle). An accumulation of β-Ala-Lys-N_ε-AMCA was always observed in GFAP positive U373-MG cells which were of astrocytic origin (Fig. 1, right). Consistent with this finding poly$(A)^+$-enriched RNA preparations from U373-MG cells contained PepT2-mRNA transcripts (Fig. 2A). The amounts of PepT2-mRNA were slightly higher in glial cells compared to U373-MG cells (Fig. 2B).

During 3 weeks of cell culture in DMEM U373-MG cells lost their ability to translocate β-Ala-Lys-N_ε-AMCA (Fig. 2A). With respect to potential substances that might influence the dipeptide transport capacity, the glioma cells were cultivated in GCM. GCM is known to be rich in small antioxidants, such as glutathione, amino acids, or neurotrophic factors like GDNF, BDNF or NGF [14,20,23,26,36]. After 3 weeks of culture in GCM U373-MG cells exhibited the same decrease of dipeptide transport as in DMEM (Fig. 2B). The transport capacity of U373-MG cells for β-Ala-Lys-N_ε-AMCA on the 2nd culture day was 1582 fmol (DMEM) and 1298 fmol (GCM) per μg protein. After 21 culture days the accumulated β-Ala-Lys-N_ε-AMCA concentrations fell below 50 fmol/μg protein in both types of media. This result was statistically significant compared to day 2 (*$p < 0.05$; Student's *t*-test). Mixed glial primary cells behaved similar (data not shown).

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