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Characterization of rPEPT2-Mediated Gly-Sar Transport Parameters in the Rat Kidney Proximal Tubule Cell Line SKPT-0193 cl.2 Cultured in Basic Growth Media

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Abstract: The rat proximal kidney tubule cell line SKPT-0193 cl.2 (SKPT) expresses the di-/tripeptide transporter PEPT2 (rPEPT2) and has been used to study PEPT2-mediated transport. Traditionally, SKPT cells have been cultured in growth media supplemented with epidermal growth factor (EGF), apotransferrin, dexamethasone, and insulin. It was recently demonstrated that omission of EGF from the culture media caused a drastic increase in the expression of rPEPT2. The hypothesis was therefore that the SKPT cell line might be able to differentiate and express rPEPT2 in the absence of the four agonists traditionally added. The aim of the study was thus to characterize Gly-Sar transport parameters in SKPT cells cultured in basic growth media (conventional media without added agonists). Morphology was studied using confocal laser scanning microscopy (CLSM) and immunohistochemistry. Monolayer integrity was evaluated using transepithelial electrical resistance (TEER) measurements and [^{3}H]-mannitol permeabilities. Di-/tripeptide transporter activity was studied using [^{14}C]-glycylsarcosine ([^{14}C]-Gly-Sar). SKPT cells grown in basic media for 4 days formed confluent monolayers with a TEER of 5.03 ± 0.33 k$\Omega\cdot$cm^2 ($n = 5$). Apical Gly-Sar uptake peaked after 3−6 days in culture. Uptake at day 4 was 5.89 ± 0.30 pmol$\cdot$cm$^{-2}\cdot$min^{-1} ($n = 3$). Di-/tripeptide uptake displayed an optimum at approximately pH 6. Affinity values for cephalexin, kyotorphin, and δ-aminolevulinic acid were comparable to those obtained in other PEPT2-expressing model systems. It can be concluded that SKPT cells grown in the absence of the agonists traditionally added to the culture media retain all necessary properties for PEPT2-mediated peptide uptake studies. Furthermore, the absence of the agonists might facilitate studies of hormonal regulation of PEPT2 expression and transport activity.

Keywords: Immunohistochemistry; Gly-Sar uptake; pH dependency; PEPT2; transepithelial transport of di-/tripeptides; SKPT cells

1. Introduction

The H^+-peptide cotransporter PEPT2 is located in the apical membrane of epithelial cells in the distal parts of the proximal kidney tubules, where it ensures the efficient reabsorption of di- and tripeptides from the ultrafiltrate.[1] Furthermore, PEPT2 is expressed in other peripheral tissues i.e. mammary gland and lungs and in central nervous tissues.[2−4] In addition to di-/tripeptides, several peptidomimetic drugs and prodrugs, including β-lactam antibiotics, valine ester prodrugs of acyclovir and gancyclovir, and the

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anticancer drug bestatin, display affinity for the transporter.[5−9] Thus, the localization and the broad substrate specificity of PEPT2 attract interest in drug distribution and elimination.

In vitro models are valuable tools for the investigation of function, expression, and regulation of di-/tripeptide transporters and have therefore been used for this purpose.[10−13] Among the in vitro models, cell lines render several advantages, i.e., the possibility to evaluate strategies for achieving drug targeting, to examine mechanisms at the molecular level, etc.[14] Continuous cell lines of epithelial origin expressing PEPT2 are the porcine kidney cell line LLC-PK1, which has previously been used for studying PEPT2 regulation[15,16] and the SKPT-0193 cl.2 (SKPT) cell line. SKPT cells have been shown to express rPEPT2 and have been used as a model to investigate rPEPT2 function and substrate affinity.[6,17−20] Traditionally, SKPT cells have been cultured in the bottom of Petri dishes, in media supplemented with the receptor agonists epidermal growth factor (EGF), insulin, dexamethasone, and apotransferrin. However, our group recently characterized Gly-Sar uptake in SKPT cell monolayers grown on permeable supports, in the traditional growth media and in media without added EGF, in order to study the EGF-mediated regulation of PEPT2.[21] We demonstrated that omission of EGF from the traditional growth media caused a drastic increase in rPEPT2 functional expression. In the present study, the aim was to characterize Gly-Sar transport parameters in SKPT cells grown in media without the supplements that have traditionally been added, henceforth termed basic media (see Materials and Methods). Permeable supports were chosen in order to allow for an estimate of basolateral uptake and transepithelial transport of di-/tripeptides. We conclude that SKPT monolayers grown in basic culture media show a rPEPT2-mediated uptake capacity which exceeds that of cells grown in the traditional media and are ideally suited for substrate-affinity experiments as well as regulatory studies on PEPT2 expression and transport activity.

2. Materials and Methods

2.1. Materials. SKPT-0193 cl.2 cells, established after SV40 transformation of isolated cells from the proximal

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tubule of rats,[22] were donated by Dr. Matthias Brandsch (Biozentrum, Halle, Germany) with kind permission of Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH). Cell culture media and Hanks' balanced salt solution (HBSS) were purchased from Life Technologies (Taastrup, Denmark). Bovine serum albumin (BSA), 2-(*N*-morpholino)-ethanesulfonic acid (MES), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonate] (HEPES), ribonuclease A, glycylsarcosine (Gly-Sar), glycylproline (Gly-Pro), cephalexin, δ-aminolevulinic acid, and kyotorphin were purchased from Sigma (Saint Louis, MO). Alexa 488-conjugated phalloidin, propidium iodide, and Alexa fluor 488-goat anti-rat IgG were supplied by Molecular Probes (Eugene, OR). Rat Anti-ZO-1 tight-junction-associated polypeptide monoclonal antibody was from Chemicon International (Temecula, CA). [^{14}C]-Glycylsarcosine ([^{14}C]-Gly-Sar) with a specific activity of 49.94 mCi·mmol^{-1} and [^{3}H]-mannitol with a specific activity of 51.50 mCi·mmol^{-1} were from New England Nuclear (Boston, MA).

2.2. Cell Culture. SKPT cells at passage 44 were seeded in culture flasks and passaged in 1:1 Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F-12 (F12) with L-glutamine, 15 mM HEPES, and piridoxine. The culture media were supplemented with 10% fetal bovine serum, 100 units·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 5 μg·mL^{-1} insulin, 4 μg·mL^{-1} dexamethasone, 5 μg·mL^{-1} apotransferrin, and 10 ng·mL^{-1} epidermal growth factor (EGF). Stock cells were subcultured every 3 or 4 days by treatment with 0.25% trypsin and 1 mM EDTA in PBS. Cells at passages 45−69 were seeded in basic culture media (DMEM/F12 media with L-glutamine, 15 mM HEPES, and pyridoxine, supplemented with 10% fetal bovine serum, 100 units·mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin), onto tissue culture treated Transwells (1 cm^2, 0.4 μm pore size) (Costar, Cambridge, MA), at a density of 5×10^4 cells·cm^{-2}. Monolayers were grown in an atmosphere of 5% CO_2−95% O_2 at 37 °C. Growth media were replaced every other day. Transepithelial electrical resistance (TEER) measurements were performed in tissue resistance measurement chambers (Endohm) with a volt ohmmeter (EVOM), both from World Precision Instruments (Sarasota, FL).

2.3. [^{14}C]-Gly-Sar Uptake and Transport Experiments. Apical media were buffered with 10 mM MES, and pH was adjusted to 6.0. Basolateral media were buffered with 10 mM HEPES and adjusted to pH 7.4. Cells were placed on a shaking plate (90 rpm), preheated to 37 °C, and allowed to equilibrate for 15 min in 0.5 mL of apical and 1 mL of basolateral buffer solution. Apical and basolateral uptake experiments as well as apical (A) to basolateral (B) and B to A transepithelial transport experiments were initiated by adding the corresponding fresh buffer containing the relevant concentration of Gly-Sar, Gly-Pro, cephalexin, kyotorphin, or δ-aminolevulinic acid and 1 μCi of [^{14}C]-Gly-Sar per mL (20.0 μM) to either the apical or basolateral side. In some experiments, 1 μCi of [^{3}H]-mannitol per mL (19.4 μM) was added to the donor solution in order to estimate the extracellular volume. Unless otherwise stated, uptake experiments were terminated after 10 min by removing the uptake medium and washing the monolayers four times with ice-cold HBSS. The polycarbonate filters were subsequently cut from the Transwell supports and placed into scintillation vials. Two milliliters of scintillation fluid was added and the radioactivity, was measured in a Packard Tri-Carb liquid scintillation counter. In A to B and B to A transepithelial flux experiments, 10 μL samples were taken from the receiver chamber at 5, 10, 20, 30, 45, and 60 min, and replaced with the corresponding buffer solutions. After 60 min, solutions were removed and the monolayers' radioactivity was measured as explained above. Fluxes of Gly-Sar were constant after 10 min. The steady-state flux values were obtained as the mean of the flux values at 20, 30, 45, and 60 min. Mannitol flux experiments were carried out using 1 μCi of [^{3}H]-mannitol per mL of donor solution. Samples (100 μL) were taken from the receiver chamber every 20 min and replaced with fresh basolateral buffer. Fluxes of mannitol were constant after 40 min. The steady-state flux values were obtained as the mean of the flux values at 60, 80, 100, and 120 min.

2.4. Kinetic Analysis. Uptake of Gly-Sar as a function of apical Gly-Sar concentration was fitted to a Michaelis−Menten type equation (eq 1):

$$V = V_{max}[S]/(K_m + [S]) \quad (1)$$

where V = uptake (pmol·cm^{-2}·min^{-1}), V_{max} = maximal uptake (pmol·cm^{-2}·min^{-1}), K_m = the Michaelis−Menten constant (μM), and [S] = Gly-Sar concentration (μM).

The IC_{50} (concentration producing half-maximal inhibition) for substrate-mediated inhibition of Gly-Sar uptake was determined using eq 2:

$$U = [(a - d)/1 + (x/c)] + d \quad (2)$$

where a = maximal uptake, d = minimal uptake (at [substrate] = ∞), $c = IC_{50}$, and U = the uptake at [substrate] = x.

The K_i (apparent affinity constant of the inhibitor) values were determined using the following equation described by Cheng and Prusoff[23]:

$$IC_{50} = K_i(1 + [S]/K_m) \quad (3)$$

where [S] and K_m refer to the substrate (Gly-Sar) concentration and to the Gly-Sar affinity constant, respectively.

2.5. pH Dependency of Gly-Sar Uptake Experiments. The pH dependency of the apical Gly-Sar uptake was determined by varying the pH of the donor solutions (range

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5.0−7.0) and maintaining the pH of the receiving solution constant. Donor solutions with pH values from 5.0 to 6.5 were buffered with 10 mM MES, and those with pH values of 7.0 were buffered with 10 mM HEPES. After 15 min of equilibration in apical (pH 6.0) and basolateral (pH 7.4) buffer solutions, the uptake experiments were performed as indicated above.

2.6. Protein Extraction and Determination. SKPT cells were lysed in lysis buffer (10 mM Tris·HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 2% NP-40), and protein content in lysates free of cellular debris was measured using Bio-Rad protein assay dye reagent concentrate (Hercules, CA), based on the Bradford method.[24]

2.7. Confocal Laser Scanning Microscopy. Cells grown on filters, as described above, were rinsed in HBSS, fixed for 10 min in HBSS with 3% paraformaldehyde, and permeabilized for 5 min in 0.1% Triton X-100 in PBS. Afterward, the cells were blocked overnight in a solution of 2% bovine serum albumin (BSA) in PBS. Cell morphology was studied by staining the actin filaments with Alexa 488-conjugated phalloidin and the cell nuclei with propidium iodide. The cells were incubated for 30 min in 200 μL of a solution of Alexa 488-conjugated phalloidin (165 nM) in PBS + 2% BSA. After being rinsed twice in PBS, they were treated with 100 μg·mL^{-1} RNAse in 2XSSC buffer solution (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 20 min. Then, the cells were counterstained with 0.5 μM propidium iodide in 2XSSC buffer solution for 5 min. ZO-1 protein localization and organization was investigated by immunostaining. After fixation and permeabilization, cells were incubated for 2 h with rat anti-ZO-1 antibody (primary antibody), used at a concentration of 1:400 in PBS plus 2% BSA. The secondary antibody was Alexa Fluor 488 goat anti-rat IgG (1:300 in PBS + 2% BSA). Cells were incubated for 90 min in the dark followed by RNAse treatment and staining with propidium iodide. All preparation steps were performed at room temperature (20 °C). Filters were mounted on coverslips, and confocal imaging was performed on a Zeiss LSM 510 confocal laser scanning microscope, using a Zeiss plan apochromat 63× oil immersion objective with a numerical aperture of 1.4. Fluorophores were excited using an argon laser line at 488 nm and a HeNe laser line at 543 nm.

2.8. Statistical Analysis. Values are given as means ± SE if the experiments were performed in several cell passages or as means ± SD if the experiments were performed in only one passage. The statistical significance of the results was determined using a two-tailed Student's *t* test. *N* is the number of individual determinations within a passage, and *n* is the number of cell passages used. $P < 0.05$ was considered significant.

3. Results

3.1. SKPT Cells Grown in Basic Culture Media Formed a Tight Monolayer. The morphology of SKPT cells grown in basic culture media was studied from 1 to 8 days after seeding. In the beginning of the culture period (days 1 and 2), flattened cells with big nuclei and a high division activity were observed (Figure 1A). The SKPT cells formed a confluent monolayer after 48 h in culture. The morphology of the monolayers remained unchanged from day 4 (Figure 1A) to day 8 (data not shown). The density of 4-day-old SKPT cells cultured in basic media was 135 ± 3 cells/~20000 μm^2 ($n = 3$). Neither detachment of the cells nor multilayer formation was observed throughout the culture period investigated. Total SKPT cell protein content as a function of culture time was measured, as described in Materials and Methods. The protein content of the SKPT cells increased rapidly in the beginning of the culture period and reached a plateau on day 3 (Figure 1B). The protein content 4 days after seeding was 0.053 ± 0.002 mg·cm^{-2} ($n = 3$).

The transepithelial electrical resistance (TEER) increased constantly during the cells' culture period and reached a value of 8.04 ± 0.13 kΩ·cm^2 ($n = 5$) 8 days after seeding (Figure 2A). In 4-day-old SKPT cells, the TEER was 5.03 ± 0.33 kΩ·cm^2 ($n = 5$, passages 64−69). The [^{3}H]-mannitol permeability decreased during the first 6 days of the culture period (Figure 2B). Mannitol permeability was $0.86 \times 10^{-4} \pm 0.29 \times 10^{-4}$ cm·min^{-1} ($n = 3$) in SKPT cells grown for 4 days at passages 43−45. The structure and localization of tight junction complexes at day 4 of growth was investigated using indirect immunofluorescence staining of the intracellular peripheral tight-junction-associated membrane protein ZO-1. ZO-1 staining (green signal) surrounded the peripheries of the cells, forming organized and continuous rings (Figure 2C).

3.2. The Apical Uptake of Gly-Sar Was Linear in the Time Range Investigated and Displayed a pH Optimum between 6.0 and 6.5. The [^{14}C]-Gly-Sar accumulation across the apical membrane of the cells and the pH dependency of the apical [^{14}C]-Gly-Sar uptake were estimated in SKPT cell monolayers grown for 4 days in basic culture media, in order to determine optimal conditions for the apical [^{14}C]-Gly-Sar uptake experiments (Figure 3). The apical [^{14}C]-Gly-Sar accumulation was measured at the lowest (25 μM) and highest (520 μM) Gly-Sar concentrations used in the experiments. The apical Gly-Sar uptake was linear over a period of 60 min in both cases (Figure 3A). When the apical uptake of [^{14}C]-Gly-Sar at a Gly-Sar concentration of 25 μM was measured at varying pH (range 5.0−7.0), the maximal Gly-Sar uptake was observed when the apical pH was between 6.0 and 6.5 and was markedly reduced by changing the pH from 6.0 to 5.0 (Figure 3B).

3.3. Apical Gly-Sar Uptake Capacity Peaked on Day 3−5 after Seeding the SKPT Cells. The transport activity of rat PEPT2 was estimated in the SKPT cells by measuring the apical uptake of [^{14}C]-Gly-Sar over a culture period of 8 days after seeding the cells (Figure 3C). The carrier-mediated component of the apical Gly-Sar uptake was estimated by performing [^{14}C]-Gly-Sar uptake experiments at a Gly-Sar concentration of 25 μM in the absence and presence of 20

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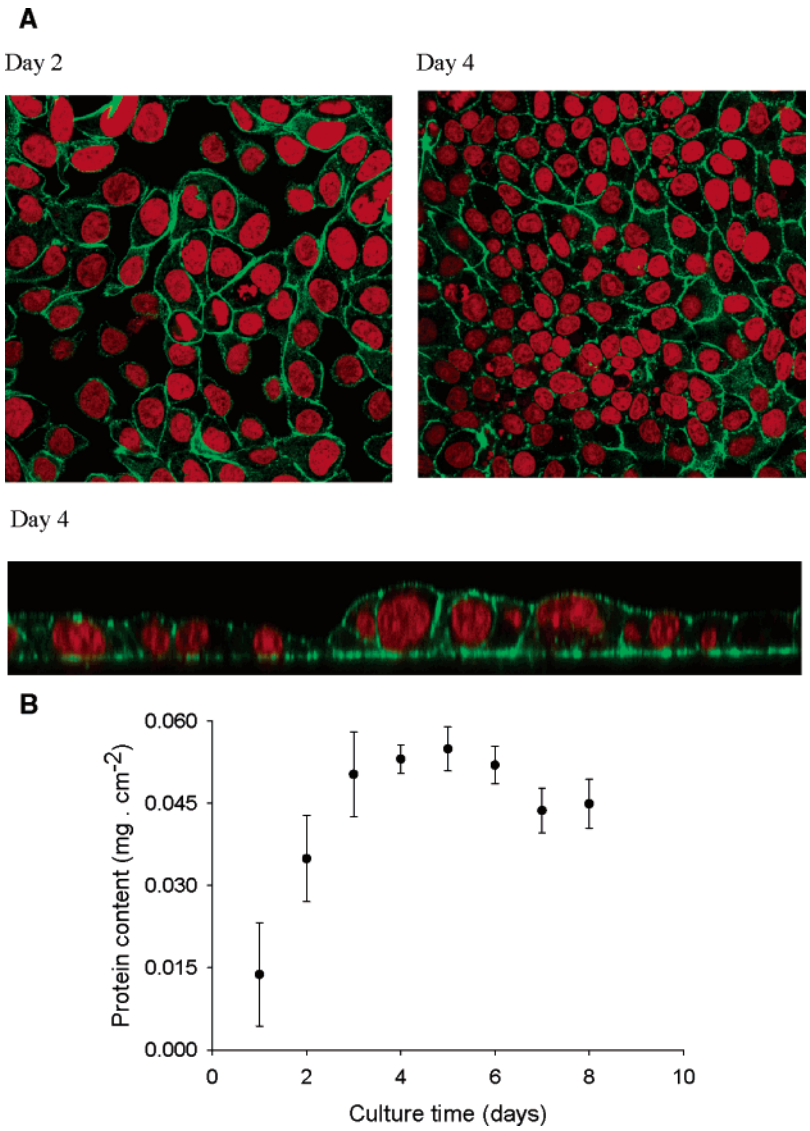

Figure 1. (A) Confocal laser scanning images of SKPT cell monolayers at different time points during the culture period. The cell nuclei were stained with propidium iodide (red signal), and Alexa 488-conjugated phalloidin was used to visualize actin filaments (green signal). Top: Horizontal sections through SKPT cell monolayers. Bottom: Vertical section through a SKPT cell monolayer (apical membrane facing upward). Each figure is representative of 3 individual preparations, each performed on different cell passages. (B) Total protein content of the SKPT cells as a function of the time in culture. Each data point represents the mean ± SE of 3 different passages. Protein determination was performed in triplicate within each passage.

mM Gly-Pro. In the beginning of the culture period (day 1 after seeding) the Gly-Sar uptake was low, but it increased quickly as the culture time of the cells increased. The maximum uptake values were observed on days 3−5, after which the Gly-Sar uptake showed a tendency to decrease (Figure 3C). The Gly-Sar uptake 4 days after seeding the SKPT cells was 5.89 ± 0.30 pmol·cm^{-2}·min^{-1} (n = 3). The presence of 20 mM Gly-Pro in the apical solution inhibited the Gly-Sar uptake throughout the culture period investigated. The Gly-Sar uptake measured 4 days after seeding the SKPT cells in the presence of 20 mM Gly-Pro was 0.18 ± 0.01 pmol·cm^{-2}·min^{-1} (n = 3). The noninhibitable Gly-Sar uptake thus represented ∼4% of the total Gly-Sar uptake measured in the absence of the inhibitor. However, when corrections for [^{14}C]-Gly-Sar present in the extracellular spaces were performed using the extracellular space marker [^{3}H]-mannitol, the Gly-Sar uptake in the presence of Gly-Pro was ∼0%, implying that all the Gly-Sar uptake was carier-mediated. Subsequent experiments were performed using SKPT cells cultured for 4 days, unless otherwise stated.

3.4. Apical Gly-Sar Uptake Displayed Michaelis−Menten Kinetics. The apical uptake of [^{14}C]-Gly-Sar was measured over a concentration range of 30−520 μM Gly-Sar in the apical solution and over a 40-min incubation period (Figure 4). The obtained [^{14}C]-Gly-Sar uptake values were corrected for extracellular radiolabeled Gly-Sar using [^{3}H]-mannitol. Corrected data were fitted to the Michaelis−Menten equation (eq 1), and kinetic constants were calculated. The kinetics of Gly-Sar uptake matched a single saturable carrier model. The apparent K_m value for Gly-Sar

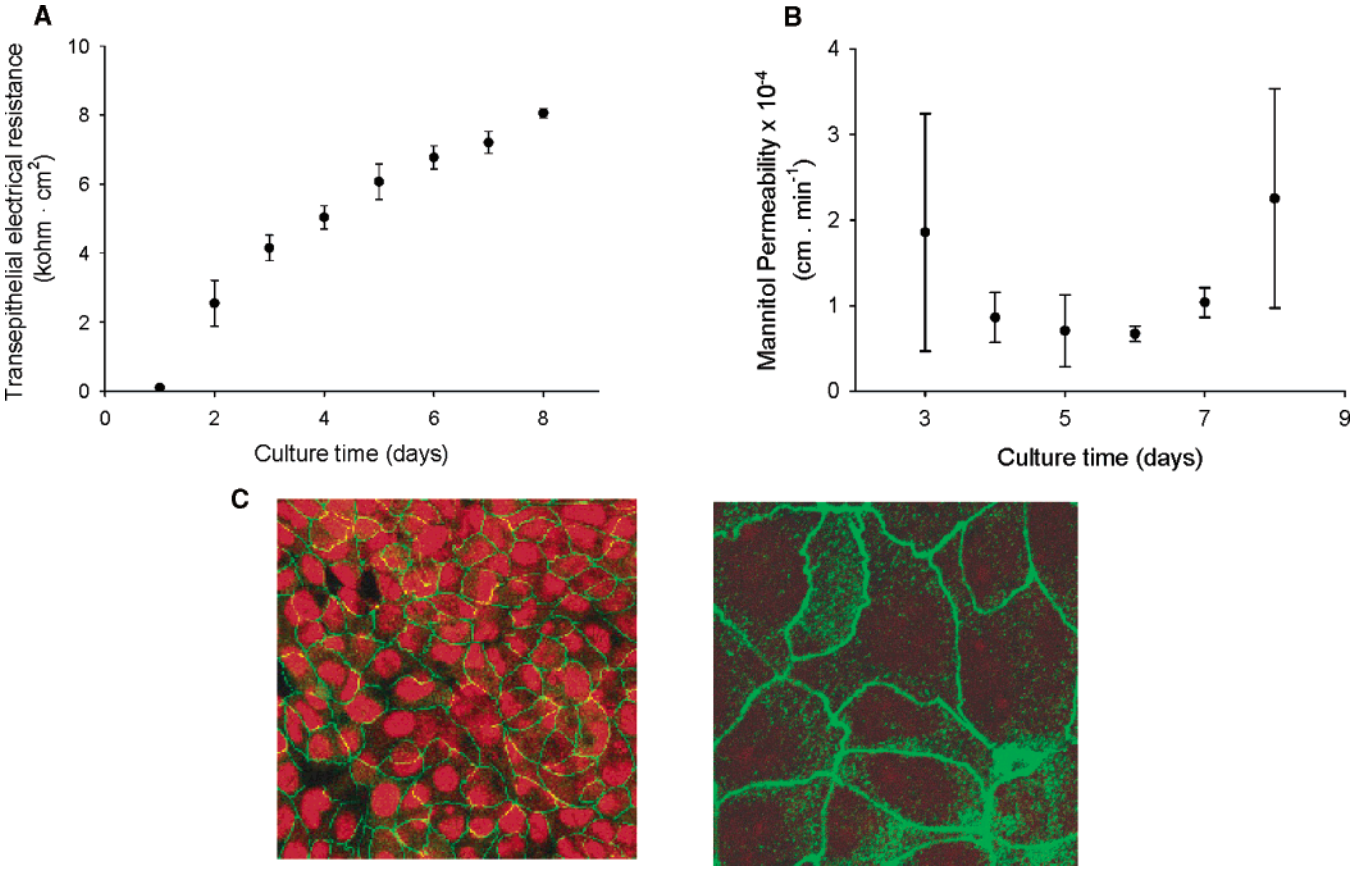

Figure 2. (A) Transepithelial electrical resistance (TEER) was investigated as a function of the culture time over a period of 8 days in SKPT cell monolayers. Each data point represents the mean ± SE of five different passages. Nine individual determinations were performed in each passage. (B) A to B permeability of [^{3}H]-mannitol (0.5 μCi per well) was investigated at different time points during the culture period in SKPT cells. Each point represents the mean ± SE of 3 different passages. Experiments were performed in triplicate within each passage. (C) Immunolocalization of ZO-1 protein in 4-day-old SKPT cell monolayers by confocal microscopy. After fixation and permeabilization, SKPT cells were incubated with rat anti-ZO-1 antibody (primary antibody). The secondary antibody was Alexa Fluor 488 goat anti-rat IgG (green signal). The cell nuclei were stained using propidium iodide (red signal). Left: Horizontal section through SKPT monolayers. Right: Horizontal section of a Z-stack (zoom = 3). Each figure is representative of 3 individual preparations.

was 159 ± 14 μM (n = 5). This value was comparable to the values obtained previously in SKPT cells grown in the traditional culture media and in media without added EGF (see Table 1). Moreover, the apparent K_m for Gly-Sar reported here is similar to the value of 129 ± 32 μM obtained in choroid plexus[25] and to that obtained in the human neuroblastoma cell line SK-N-SH transiently transfected with rat PepT2 cDNA (~85 μM).[26] The maximal capacity V_{max} was 21.23 ± 4.01 pmol·cm^{-2}·min^{-1} (n = 5).

3.5. The Apical Uptake of Gly-Sar in SKPT Cells Was Inhibited by Cephalexin, δ-Aminolevulinic Acid and Kyotorphin in a Dose-Dependent Manner. The affinity of cephalexin, kyotorphin, and δ-aminolevulinic acid was determined by their ability to inhibit the apical uptake of [^{14}C]-Gly-Sar. The apical uptake of [^{14}C]-Gly-Sar was measured over an inhibitor concentration (0−500 μM) range, over a 40-min incubation period, in 4-day-old SKPT cells grown in basic culture media. Data were fitted to eq 2, and IC_{50} values were calculated. K_i values were then estimated using the equation described by Cheng and Prusoff (eq 3). The apical uptake of Gly-Sar was inhibited in a dose-dependent manner by the compounds studied. The affinity contants (K_i) were 49 ± 7 μM, 29 ± 2 μM, and 231 ± 91 μM, and the maximal inhibition of the Gly-Sar uptake was 99 ± 0.2%, 90 ± 2%, and 86 ± 3% for cephalexin, kyotorphin, and δ-aminolevulinic acid, respectively (n = 4).

3.6. Transepithelial (A to B) Gly-Sar Flux Is Mainly via a Passive Pathway. The transepithelial apical (A) to basolateral (B) flux of 20 μM of [^{14}C]-Gly-Sar across the monolayer was measured in 4-day-old SKPT cells in the absence and presence of 2 mM Gly-Pro in the apical solution (Figure 5A). The A to B [^{14}C]-Gly-Sar flux in the absence of Gly-Pro (6.73 ± 0.06 pmol·cm^{-2}·min^{-1}, n = 7) was

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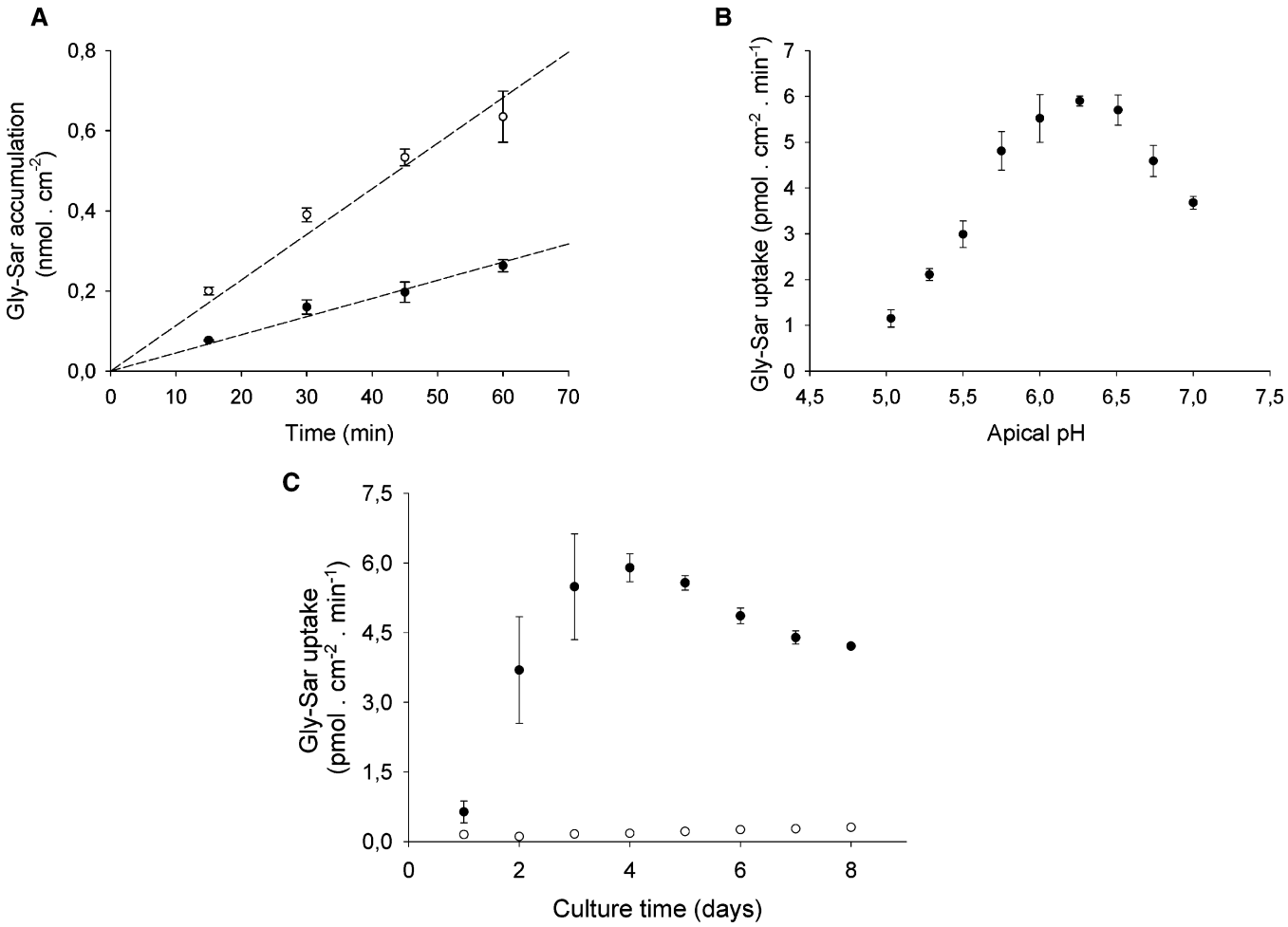

Figure 3. (A) Cellular uptake across the apical membrane of [^{14}C]-Gly-Sar at an extracellular Gly-Sar concentration of 25 mM (●) and 520 mM (○) as a function of the incubation time in SKPT cells cultured for 4 days in basic media. Each data point represents the mean ± SD of 3 different wells. (B) pH dependence of apical Gly-Sar uptake in 4-day-old SKPT cells. Uptake of [^{14}C]-Gly-Sar at a Gly-Sar concentration of 25 μM was measured at varying apical pH after 10 min of incubation. The pH of the basolateral solution was 7.4 in all the experiments. Each data point represents the mean ± SD of 4 different wells. (C) Influence of culture time on Gly-Sar uptake in SKPT cells. Uptake of [^{14}C]-Gly-Sar at a Gly-Sar concentration of 25 μM was measured over 10 min of incubation in the absence (●) and presence (○) of 20 mM Gly-Pro. Each data point represents the mean ± SE of 3 individual passages. Experiments were performed in triplicate within each passage.

Table 1. Characteristics of SKPT Cells Grown under Different Culture Conditions

	traditional media on plastic dishes	traditional media	traditional media without EGF	basic media[a]
cells/area (cells/~20000 mm^2)	nd[b]	256 ± 24[c]	173 ± 20[c]	135 ± 3
protein content (mg·cm^{-2})	~0.10[d]	0.25 ± 0.02[c]	0.28 ± 0.05[c]	0.053 ± 0.002
TEER (kΩ·cm^2)	nm[e]	~3[f]	~7[f]	4.93 ± 0.28
noninhibitable component[g]	18%[h]	40%[f]	9%[f]	4%
K_m[i] (μM)	67 ± 2[d]	114 ± 8[c]	90 ± 3[c]	159 ± 14
V_{max}[i] (pmol·cm^{-2}·min^{-1})	~15[d]	9.02 ± 1.16[c]	34.56 ± 4.14[c]	21.23 ± 4.01

[a] Basic media: Traditional media without added agonists. [b] Not determined. [c] Reference 21. [d] Reference 18. [e] Not measurable. [f] Data not previously published. [g] Estimated as % residual Gly-Sar uptake at an apical Gly-Sar of 500 μM in the presence of a surplus of Gly-Pro. [h] Reference 19. [i] Apparent affinity constant (K_m) for Gly-Sar and maximal capacity (V_{max}) of the apical Gly-Sar uptake.

significantly higher than the flux obtained in the presence of the inhibitor (6.03 ± 0.13 pmol·cm^{-2}·min^{-1}, $n = 7$) ($P < 0.01$). The carrier-mediated component of the transepithelial (A to B) flux of [^{14}C]-Gly-Sar was, however, only ~10% of the total flux. The [^{14}C]-Gly-Sar taken up by the SKPT cells was determined after the transport experiment,

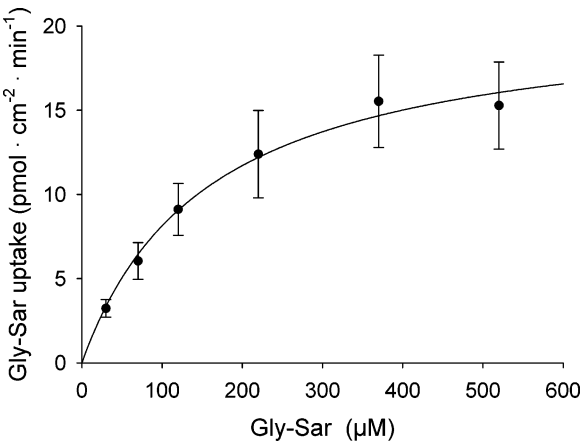

Figure 4. Gly-Sar concentration dependence of apical uptake of [^{14}C]-Gly-Sar was measured in SKPT cells grown for 4 days in basic culture media over a 40-min incubation period. Corrections for [^{14}C]-Gly-Sar remaining in the extracellular spaces were performed as indicated in Materials and Methods. The corrected data points were fitted to the Michaelis−Menten equation. Each data point represents the mean ± SE of 5 individual passages. Experiments were performed in duplicate within each passage.

both in the absence and in the presence of Gly-Pro. The uptake of [^{14}C]-Gly-Sar across the apical membrane in SKPT cells was 0.23 ± 0.004 nmol·cm^{-2}·(60 min)$^{-1}$ (n = 3) when measured in the absence of Gly-Pro. When 2 mM of Gly-Pro was present in the donor solution, the [^{14}C]-Gly-Sar uptake was 0.01 ± 0.001 nmol·cm^{-2}·(60 min)$^{-1}$ (n = 3) and after performing corrections for [^{14}C]-Gly-Sar present in extracellular spaces, the [^{14}C]-Gly-Sar accumulation was again not significantly different from zero.

The transepithelial B to A flux of 20 μM of [^{14}C]-Gly-Sar in 4-day-old SKPT cells was also studied and compared with the A to B flux across the monolayers (Figure 5B). The transepithelial A to B and B to A fluxes were 11.39 ± 4.93 pmol·cm^{-2}·h^{-1} (n = 3) and 10.47 ± 4.01 pmol·cm^{-2}·h^{-1} (n = 3), respectively. Although the A to B transepithelial flux was modestly higher than the flux in the opposite direction, these values were not significantly different (P > 0.05).

The apical and basolateral [^{14}C]-Gly-Sar uptake were studied at a [^{14}C]-Gly-Sar concentration of 20 μM, over 10 min of incubation, in SKPT cells grown for 4 days in basic media (Figure 5C). The relatively short incubation period of 10 min was chosen in order to avoid a significant component of transported radiolabeled Gly-Sar from entering the cells via the apical peptide transporter. The apical Gly-Sar uptake was estimated to 2.37 ± 0.39 pmol·cm^{-2}·min^{-1} (n = 5). The basolateral Gly-Sar uptake (0.50 ± 0.11 pmol·cm^{-2}·min^{-1}, n = 5) was significantly lower than the rPEPT2-mediated Gly-Sar uptake (P < 0.01).

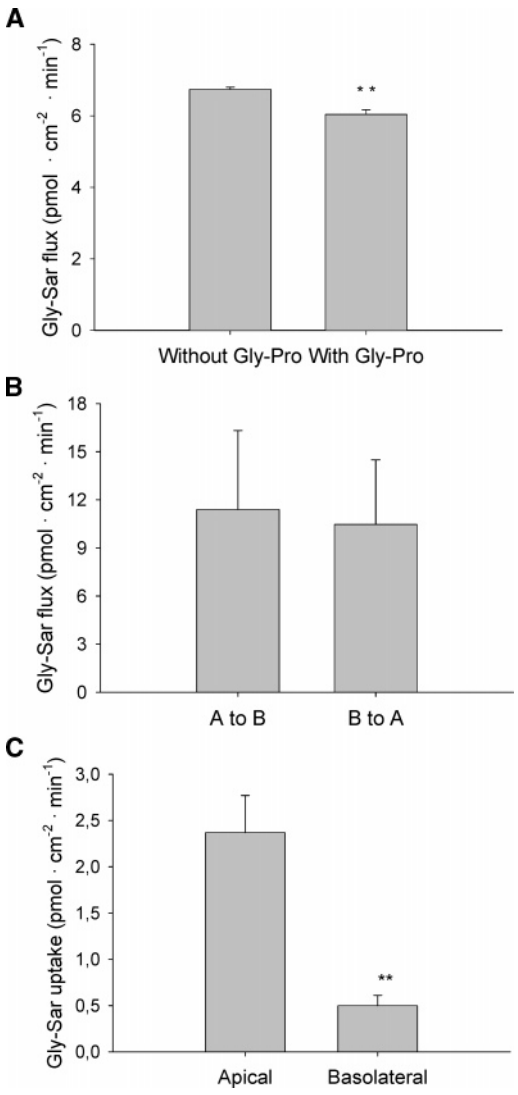

Figure 5. (A) Apical (A) to basolateral (B) transepithelial flux of 20 μM [^{14}C]-Gly-Sar was investigated in 4-day-old SKPT cells in the absence and presence of 2 mM Gly-Pro. Each bar represents the mean ± SE of 7 different passages. Experiments were performed in triplicate within each passage. The [^{14}C]-Gly-Sar fluxes in the absence and presence of Gly-Pro were significantly different. **P < 0.01 (B) A to B and B to A transepithelial flux of 20 μM [^{14}C]-Gly-Sar was investigated in 4-day-old SKPT cell monolayers. Each bar represents the mean ± SE of 3 different passages. Experiments were performed in triplicate within each passage. (C) Apical and basolateral uptake of 20 μM [^{14}C]-Gly-Sar was investigated in 4-day-old SKPT cells over 10 min of incubation. Each bar represents the mean ± SE of 5 different passages. Experiments were performed in duplicate within each passage. The basolateral [^{14}C]-Gly-Sar uptake was significantly lower than the apical uptake. **P < 0.01. In all experiments apical and basolateral pH were 6.0 and 7.4, respectively.

4. Discussion

4.1. SKPT Cells Grown in Basic Media Formed Tight Confluent Monolayers Displaying Apical Carrier-Mediated Gly-Sar Uptake. SKPT cells grown in basic culture media formed confluent monolayers. A lower cell density was, however, observed in these monolayers when compared with SKPT cells grown in the traditional media or in media without added EGF (Table 1). The total protein content of the 4-day-old SKPT cells was also considerably lower than

the one obtained previously for SKPT cells grown in the traditional culture media and in EGF-free media[21] (Table 1), which may be partially related to the lower cell density observed in the SKPT monolayers grown in the basic culture media (Table 1), i.e., the presence of fewer but bigger cells implies less tight junctional area per membrane area. The transepithelial electrical resistance (TEER), usually related to the degree of differentiation of epithelial cells, increased continuously during the growth period studied and was accompanied by the decrease in mannitol permeability observed from day 3 to day 6, indicating that the rise in TEER may be caused by a decrease in the paracellular permeability. The patterns observed may, however, change at different cell passage numbers. Staining of the tight-junction-associated protein ZO-1 showed well-developed, continuous tight-junction structures on day 4 of growth, which suggests the formation of polarized and differentiated monolayers. Investigation of functional Gly-Sar transport parameters showed that its apical uptake was solely carrier-mediated, maximal at day 3−5, and with a pH optimum similar to the one observed by Brandsch et al. in SKPT cells grown in the traditional media.[18] Transepithelial Gly-Sar flux was mainly passive, with only a small Gly-Pro inhibitable component.

4.2. Comparison of SKPT Cells Grown under Different Culture Conditions. The maximal capacity V_{max} of the apical Gly-Sar uptake in SKPT cells cultured in basic media was considerably higher than the one obtained previously in cells cultured in the traditional media (Table 1). The fact that PEPT2 function is retained in the absence of the four agonists indicates that addition of these to the culture medium is not a prerequisite for cell growth and PEPT2 expression. Indeed, the higher V_{max} found in cells grown without agonists may be advantageous for di-/tripeptide transport studies. A lower V_{max} value was, however, observed in SKPT cells grown in basic media if compared to cells grown in traditional culture media without EGF, that is to say in the presence of insulin, apotransferrin, and dexamethasone (Table 1). The experiments performed to determine the kinetic constants in SKPT cells cultured in the different growth media were, however, carried out at different cell passages and with different parental cell populations. This may explain the differences in V_{max}. Nevertheless, a possible upregulation of rPEPT2 by some of the agonists remaining in the culture media cannot be discarded. To our knowledge, no reports have been published regarding PEPT2 regulation with apotransferrin, dexamethasone, or insulin. However, PEPT2 regulation seems plausible since insulin and dexamethasone upregulation of the low-affinity counterpart, PEPT1, has previously been demonstrated.[27−29] Thus, whether the higher V_{max} observed in SKPT cells grown in EGF-free media, but in the presence of insulin, apotransferrin, and dexamethasone, is due to rPEPT2 regulation or to interpassage variations is not known. Further studies will therefore be necessary in order to determine this.

The absence of tonic stimulation from EGF, insulin, apotransferrin, and dexamethasone may also be useful in studies of di-/tripeptide transporter regulation by these agonists as well as other hormones and growth factors. Furthermore, the so-called noninhibitable component of di-/tripeptide uptake, which is essentially due to isotope present in the extracellular spaces, is low when SKPT cells are grown in basic media (due to a combination of high di-/tripeptide uptake capacity and low extracellular space) (Table 1), making corrections less necessary. Moreover, the PEPT2 affinity values for kyotorphin, cephalexin, and δ-aminolevulinic acid obtained in this study using the SKPT cells grown in basic culture media are comparable to the values previously reported in the literature when using other assay systems (Table 2).[30−36] Altogether, SKPT cells grown in basic culture media may serve as a useful standard model for di-/tripeptide uptake studies.

4.3. Transepithelial Gly-Sar Transport in SKPT Cells Grown in Basic Media Is Mainly Paracellular. Transepithelial Gly-Sar flux was observed in SKPT cell monolayers. However, the carrier-mediated component of the flux was

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Table 2. Affinity Constant Values of Kyotorphin, δ-Aminolevulinic Acid, and Cephalexin Obtained from Gly-Sar Inhibition Studies in SKPT Cells Grown in Basic Media and in Other Assay Systems

	PEPT2 substrate affinity values reported in the literature			
	affinity constant (μM)	assay system	ref	K_i (μM) SKPT cells
kyotorphin	30 ± 4 (K_i)	synaptosomes from rat cerebellum	30	29 ± 2
	∼5 (K_i)	rat choroid plexus	25	
	29 ± 14 (K_i)	synaptosomes from rat cerebral cortex	32	
δ-aminolevulinic acid	226 ± 16 (K_m)	rabbit PEPT2 expressing *Pichia pastoris*	33	231 ± 91
	227 ± 29 (K_m)	rabbit PEPT2 expressing *Xenopus laevis* oocytes	33	
	260 ± 14 (K_m)	rat choroid plexus	34	
cephalexin	68.2 ± 5.2 (K_i)	SKPT cells: traditional media	6	49 ± 7
	35.2 (K_i)	SKPT cells: media without EGF	35	
	49 (K_i)	rPEPT2 expressing LLC-PK1 cells	36	

very low. Therefore, the Gly-Sar transepithelial flux across the SKPT cell monolayers is most likely flux occurring through the paracellular pathway. Contrarily, Shu et al. observed transepithelial transport of fosinopril in the A to B direction.[37] Experiments were, however, performed between 18 and 21 days after seeding the SKPT cells in traditional media with agonists, and the results are consequently not readily comparable. Furthermore, fosinopril may not be exclusively transported by rPEPT2 and other transporters could therefore be responsible for the carrier-mediated A to B flux. Neumann et al., who also worked with cells grown in traditional culture media, reported Gly-Sar net flux (∼20% of the total A to B flux) in the A to B direction.[38] The A to B flux was, however, not inhibited by alafosfalin, a competitive inhibitor for Gly-Sar. In order to display active transepithelial transport, cells should exhibit apical and basolateral transport activity. Several groups working with different tissue models suggested the presence of a basolateral peptide transporter in the nephron. Sugawara et al. used renal basolateral membrane vesicles prepared from rat proximal tubules and reported a K_m value for the uptake of Gly-Sar in the millimolar range (8 mM).[39] The presence of a basolateral peptide transporter with a K_m value for Gly-Sar of ∼70 μM was also demonstrated in Madin-Darby canine kidney (MDCK) cells.[40,41] Regardless of the characteristics of the basolateral transport system for Gly-Sar, its activity in the SKPT cells at the Gly-Sar concentration used in flux experiments was estimated. This activity was almost 5 times lower than the activity observed for PEPT2 at the same Gly-Sar concentration (and not different from the basolateral transport activity in cells grown in traditional media, data not shown). Therefore, the low basolateral Gly-Sar transport activity may limit the carrier-mediated transepithelial flux of Gly-Sar in the SKPT cells. Furthermore, the basolateral peptide transporter does not seem to be regulated by the agonists present in the traditional medium. Whether the SKPT cell line presents characteristics similar to those of the proximal kidney tubules in terms of peptide transport parameters is not known. It must be noted, however, that naturally occurring di-/tripeptides are hydrolyzed in the cytosol of the proximal tubule cells and may thereby cross the basolateral membrane via the action of amino acid transporters. From the physiological point of view there is consequently no apparent need for a basolateral peptide transport system. However, other transport systems may be participating in the basolateral transport of peptides.

In conclusion, SKPT cells grown in basic culture media appear to be a useful cell model system for investigating PEPT2-mediated di-/tripeptide uptake phenomena. The culture conditions ensure high functional PEPT2 expression, and the absence of high concentrations of agonists should in theory make regulatory studies more feasible. Whether

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the low transepithelial carrier-mediated flux is a property of SKPT cells or whether it is representative for proximal kidney tubule epithelia remains to be studied.

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