Amino Acids (2010) 38:753–761
DOI 10.1007/s00726-009-0280-5

ORIGINAL ARTICLE

L-Proline transport into renal OK epithelial cells: a second renal proline transport system is induced by amino acid deprivation

A. Nickel · U. Klein · D. Weitz · H. Daniel

Received: 8 August 2008 / Accepted: 13 March 2009 / Published online: 31 March 2009

Abstract Influx of [^{3}H]-L-proline into renal OK cells revealed that basal transport was mediated by the transporter SIT1. When cells were submitted for 8 h to amino acid deprivation, uptake of L-proline was now dominated by a low-affinity system with an apparent K_m of 4.4 ± 0.6 mM and a V_{max} of 10.2 ± 0.6 nmol/mg of protein/min operating in addition to the high-affinity SIT1 system with a K_m of 0.12 ± 0.01 mM and a V_{max} of 0.28 ± 0.04 nmol/mg of protein/min. The low- and high-affinity proline transporting systems were sensitive to inhibitors of JNK and PI-3 kinases, whereas a GSK-3 inhibitor affected only the upregulated transport system. Ion-replacement studies and experiments assessing substrate specificities for both systems provided strong evidence that SNAT2, that showed two- to threefold increased mRNA levels, is the responsible transporter mediating the increased proline influx under conditions of amino acid deprivation.

Keywords Transport · Proline · Amino acid deprivation · Regulation · SIT1 · SNAT2

Introduction

To prevent loss of amino acids via urine, efficient renal reabsorption in the proximal tubule is achieved by a concerted action of several amino acid transporting systems in the apical membrane of epithelial cells. There are six major epithelial L-proline transporting systems that have been characterized so far from mammalian renal tissues and these are SIT1, PAT1, PAT2 and SNAT2, B^0AT1 and B^0AT2. The groups of Hediger as well as of Bröer cloned and characterised the system IMINO from rat which was designated as SIT1 (Sodium/Imino acid Transporter 1) and from mouse kidney, designated as IMINO^B (Kowalczuk et al. 2005; Takanaga et al. 2005). Humans have only one IMINO gene, whereas mouse and rat have two homologue IMINO genes. SIT1/IMINO^B is a member of the Na^+ and Cl^--dependent neurotransmitter transporter family SLC6 and is encoded by the slc6a20 gene. SIT1 is highly expressed in epithelial cells of intestine, kidney tubular S3 segments and choroid plexus (Takanaga et al. 2005). IMINO^B was mainly found in brain, kidney, small intestine, thymus, spleen and lung (Kowalczuk et al. 2005). IMINO is Na^+ and Cl^--dependent and high-affinity substrates are L- and D-proline, hydroxyproline, betaine as well as D- and L-pipecolate with K_m values in the range of 0.1–0.5 mM. Neutral amino acids like cysteine, leucine, isoleucine, phenylalanine, valine and alanine are poor substrates, cationic amino acids, anionic amino acids and glycine are not transported.

PAT1 and PAT2 are members of the SLC36 family of proton-coupled transporters expressed in epithelial cells. PAT1 prefers amino acids with short side-chains and pharmacologically active proline-derivatives such as *cis*-4-hydroxy-D-proline (Boll et al. 2002; Kennedy et al. 2005). PAT1 mRNA is highly expressed in small intestine, colon, kidney and brain. PAT2 is mainly found not only in heart and lung, but also in kidney, testes, liver and spleen. However, PAT2 is in comparison to PAT1 a high-affinity transporter with 10–30 times higher affinities for the identical substrates but has in general a narrower substrate

A. Nickel · U. Klein · D. Weitz · H. Daniel (✉)
Molecular Nutrition Unit, Technical University of Munich,
Am Forum 5, 85350 Freising-Weihenstephan, Germany
e-mail: daniel@wzw.tum.de

specificity (Boll et al. 2002). PAT1 is found in epithelial cell membranes (Brandsch 2006) and recently Broer et al. (2008) demonstrated the localisation of PAT2 in brush-border membranes of the proximal tubule.

SNAT2 represents a neutral amino acid transporter of the *SLC38* gene family with system A specificity and is highly regulated upon alterations of intracellular concentrations of organic osmolytes (Bode and Kilberg, 1991). SNAT2 is Na^+-dependent, which prefers not only alanine, serine, cysteine, glutamine and asparagine but also accepts proline as substrate (Hatanaka et al., 2000). SNAT2 function and regulation by amino acid restriction has been extensively studied in rat skeletal muscle and adipose tissue and in L6 myotubes and 3T3-L1 adipocytes (Hyde et al. 2001, 2002). However, SNAT2 is also found in apical membranes of polarized cells and is subject to adaptive changes here as well (Jones et al. 2006; Thongsong et al. 2005).

B^0AT2 as a member of the SLC6 family has been cloned from mouse (Broer et al. 2006). It is a Na^+-dependent, but Cl^--independent transporter, which prefers branched-chain amino acids and proline with affinities ranging from 40 to 200 μM. RT-PCR experiments showed expression of mouse B^0AT2 in brain, lung and kidney, the localisation of the protein in kidney cells was not yet reported. Furthermore, proline is also transported—although with low affinity—by the amino acid transporter B^0AT1, which is expressed at the apical membrane of intestinal and renal epithelial cells (Bohmer et al. 2005).

Here, we have employed opossum kidney cells as a well-characterised renal epithelial cell line to characterize proline transport under basal conditions and after the cells have been exposed to amino acid deprivation. Expression of SIT1 in OK cells was recently shown by Ristic et al. (2006). Heterologous expression of oSIT1 in oocytes exhibited a high-affinity for L-proline exceeding that of isoleucine almost tenfold, whereas maximal velocity was threefold lower for proline flux compared to isoleucine influx (Ristic et al. 2006). Therefore, oSIT1 seems to differ from the mammalian SIT1 class transporters and resembles more the characteristics of a Na^+-dependent B^0-type. However, Hediger and Bröer reported for mammalian SIT1 that L-proline transport could, to some extent, be inhibited by neutral amino acids although these were not efficiently transported (Kowalczuk et al. 2005; Takanaga et al. 2005).

Materials and methods

Opossum kidney (OK) cells were kindly provided by H. Murer (University of Zurich, Switzerland). Media and supplements for cell culture were obtained from PAA, Pasching, Austria. Cell culture plates were from Renner (Dannstadt, Germany). RNAWIZ and the Retroscript-Kit were purchased from Ambion (Kassel, Germany). All chemicals with the highest purity were from Roth (Karlsruhe, Germany). L-[2,3-^{3}H] Proline (42 Ci/mmol), L-[^{3}H-G] Glutamate (49 Ci/mmol), L-[4,5-^{3}H] Isoleucine (94 Ci/mmol), L-[4,5-^{3}H] Lysine (99 Ci/mmol), [2-^{3}H] Glycine (21 Ci/mmol) and L-[2,3-^{3}H] Alanine (47 Ci/mmol) were from Amersham (Freiburg, Germany), non-labelled amino acids and pharmaceuticals were from Sigma (Deisenhofen, Germany).

Cell culture

Opossum kidney cells were used between passage 40 and 60. Cells were cultured and passaged in DMEM/F-12 supplemented with 10% fetal calf serum and 1% Penicillin/1% Streptomycin. The cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged at pre-confluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA. The cells were seeded with a starting density of 0.5×10^6 per well of a 6-well plate. For amino acid-free incubations, cell monolayers were washed once in buffer contained 25 mM Hepes/Tris pH 7.4, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose and incubated in the same solution for the indicated periods.

Amino acid uptake assay

Opossum kidney cells were grown on 6-well plates, and standard transport assays were performed on the first to second day after cells reached confluency. Immediately before the measurements, cell monolayers were washed once with the buffer used for amino acid deprivation. Uptake was initiated by adding 1 ml of the same buffer now containing the radiolabelled substrate, 24 nM L-[2,3-^{3}H] Proline, 21 nM, L-[2,3-^{3}H] Alanine, 48 nM [2-^{3}H] Glycine, 11 nM L-[4,5-^{3}H] Isoleucine, 10 nM L-[4,5-^{3}H] Lysine or 20 nM L-[^{3}H-G] Glutamate. Cell monolayers were incubated with substrates for 6 min as during this time uptake was linear over time. Uptake was terminated by the removal of the test solution followed by three times washing with ice-cold uptake buffer. Afterwards, the cells were solubilised with 1 ml Igepal-lysis-buffer (50 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1.5 mM $MgSO_4$, 0.5% Igepal CA-630) and radioactivity was determined by liquid scintillation counting. Protein determination according to the procedure of Bradford (Bradford 1976) was performed in two parallel samples not subjected to the transport assay.

For Na^+-free buffer conditions, NaCl was replaced by equimolar choline chloride, for Cl^--free buffer, Cl^- was replaced by equimolar D-gluconate, for Li^+-containing buffer NaCl was replaced by equimolar LiCl and for buffer

pH 6.0 Hepes/Tris was replaced by equimolar Mes/Tris pH 6.0. Solutions containing actinomycin D, cycloheximide, SB-415286, wortmannin or JNK inhibitor II also contained 0.5–1% dimethylsulfoxide (DMSO) with controls also exposed to the same DMSO concentrations. Other variations of these standard assay conditions are given in the text or figure legends.

ATP levels in OK cells were determined using an ApoSENSOR™ Cell Viability Assay Kit. Cells were trypsinized, cell numbers were determined and 1,000 cells were used for analysis. The manufacturer's protocol was modified by using the double concentration of releasing buffer for a more efficient release of ATP from the cells. Luminiscence was measured using a multiwell-plate reader (Luminoskan Ascent, Labsystems).

Quantitative real-time PCR for mRNA determination

Total RNA was isolated with RNAwiz. RNA concentration was determined by UV spectroscopy at 260 nm. One microgram of total RNA was used for cDNA synthesis in a final volume of 40 µl. RNA was heated to 65°C for 5 min with fivefold M-MLV Reverse Transcriptase Reaction Buffer and 300 µM each of dATP, dGTP, dCTP, and dTTP, and subsequently cooled on ice for 5 min. First strand DNA synthesis was accomplished with 0.08 µg random hexamer primers, 12.5 U of RNase inhibitor, and 200 U of M-MLV Reverse Transcriptase. The completed reverse transcription mixture was preincubated for 10 min at room temperature. PCR was carried out for 50 min at 42°C and a final step of 15 min at 70°C was performed to inactivate the reaction. Samples were cooled and stored at −20°C until use.

Primer design for LightCycler (Roche Applied Sciences)-based quantitative real-time PCR was done with regard to primer dimer formation, selfpriming formation, and primer melting temperature using the LightCycler Probe Design Software (Roche). BLAST search on the Monodelphis domestica (opossum) genome at the Ensemble database (www.ensembl.org) revealed that primers are gene-specific and if possible, those primers, which span at least one intron, were chosen. Primers of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. Based on these criteria, the following primers (MWG, Ebersberg, Germany) were used (1) for SIT1 amplification: forward primer 5′-TGCCTACCCAACGGAA-3′ and reverse primer 5′-GCGGCTTGAAATGGTG-3′, corresponding to residues 1026–1011 and residues 1281–1266, respectively, of the published cDNA sequence; (2) for GAPDH amplification: forward primer 5′-ATTACCGCTACCCAGAAG-3′ and reverse primer 5′-GGTGTCGCTGTTAAAGTCA-3′, corresponding to residues 512–494 and residues 841–823, which yield a 348-bp product; (3) for SNAT2 amplification: forward primer 5′-CTGGTTTCAGTGGTGCT-3′ and reverse primer 5′-CGGGGTTCACAGGAATC-3′, corresponding to residues 604–588 and residues 842–826, which yield a 255-bp product.

Each PCR reaction was conducted with 25 ng reverse transcribed total RNA (cDNA-RNA hybrid) in a final volume of 10 µl consisting, furthermore, of 4 mM $MgCl_2$, 0.4 µM forward and reverse primers, and tenfold FastStart DNA Master SYBR Green I (containing "Hot Start" Taq DNA polymerase, reaction buffer, dNTP mix, $MgCl_2$, and SYBR Green I dye). After initial denaturation at 95°C for 10 min, amplification was performed by 50 cycles (GAPDH) or 45 cycles (SNAT2, SIT1) using the following parameters: 95°C for 15 s (denaturation), 63°C (SIT1, SNAT2) or 60°C (GAPDH) for 10 s (annealing), 72°C for 20 s (elongation), and 82°C (SIT1), respectively, for 5 s (melting primer dimers prior to quantification). A negative control without cDNA was also performed for 50 and 45 cycles, respectively, to uncover possible contamination. The relative amount of target mRNA normalised to a reference gene (housekeeping gene) was calculated according to the following formula (Pfaffl et al. 2002):

$$\mathrm{RF\,(regulation\ factor)} = 2^{\Delta C}T^{\mathrm{target(control-sample)}} / 2^{\Delta C}T^{\mathrm{reference(control-sample)}}$$

Expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalisation.

Calculations and Statistics

All calculations were performed using Prism 4 software (GraphPad Software, Los Angeles, CA). For each variable, at least three independent experiments were carried out. Data are given as the mean ± SEM. Statistically significant differences ($p < 0.05$) were determined with the Student's t test for non-paired data.

Results

Basal and amino acid deprivation-induced L-proline transport

Basal L-proline transport into OK monolayers was Na^+ and Cl^--dependent since replacement of either Na^+ or Cl^-inhibited almost 90% of proline influx (Table 1). To assess the specificity of transport function in OK cells, we measured proline transport in the absence and the presence of prototypical amino acids for SIT1, B^0AT1, B^0AT2 and the PAT systems. Imino acids typically serving as high-affinity SIT1 substrates caused transport inhibition up to 90% (Table 2). More hydrophobic amino acids that are

Table 1 Na^+- and Cl^--dependence of basal L-proline uptake

Uptake buffer containing	[^{3}H]-L-proline uptake pmol/mg of protein/min
NaCl	0.054 ± 0.03
Choline–Cl	0.007 ± 0.001
Na–gluconate	0.007 ± 0.001
LiCl	0.011 ± 0.001

Tracer L-proline uptake into OK cells grown under standard conditions with either NaCl in the uptake medium or when Na^+ was replaced by choline or Li^+ and Cl^- replaced by gluconate. Data are mean ± SEM ($n = 4$)

Table 2 Transport of proline in the absence or the presence of prototypical substrates for SIT1, $B^0AT1/2$ and PAT 1/2

Substrate	Relative L-proline uptake (%)
[^{3}H]-L-proline	100 ± 3
Typical SIT1-substrates	
D-Proline (10)	18 ± 1
L-Pipecolate (10)	10 ± 1
D-Pipecolate (10)	22 ± 2
N-Methylproline (10)	12 ± 1
α-N-MeAIB (10)	10 ± 1
Sarcosine (10)	11 ± 1
Betaine (10)	14 ± 1
N,*N*-Dimethylglycine (10)	13 ± 4
Nipecotic acid (10)	27 ± 1
Isonipecotic acid (10)	22 ± 1
Typical $B^0AT1/2$-substrates	
L-Alanine (1)	79 ± 2
L-Phenylalanine (1)	67 ± 2
L-Leucine (1)	86 ± 1
L-Isoleucine (1)	71 ± 5
L-Methionine (1)	68 ± 2
L-Cysteine (1)	73 ± 1
L-Glutamine (10)	50 ± 5
Typical PAT1-substrates	
Taurine (10)	82 ± 3
GABA (10)	95 ± 2
Vigabatrin (5)	93 ± 1
Typical PAT1/2-substrates	
β-Alanine (10)	68 ± 1
L-Cycloserine (10)	83 ± 4
D-Cycloserine (10)	86 ± 2

Tracer L-proline uptake was determined in the absence or the presence of the selected competitors in cells grown under standard conditions. Substrates/competitors were used in the concentration (in mM) indicated in parentheses. Data are mean ± SEM ($n = 3$–4)

substrates typical for B^0AT1 and B^0AT2, including alanine, leucine, isoleucine, methionine, phenylalanine and cysteine also inhibited proline uptake but only by 14–33% (Table 2). Glutamine-reduced proline influx to 50% at a concentration of 10 mM. As compared to the imino acids, these hydrophobic amino acids and glutamine may be considered low-affinity type substrates. When typical PAT-substrates such as β-alanine, taurine, cycloserine (L- and D-), vigabatrin and GABA were employed as competitors, L-proline transport was not significantly affected (Table 2). Analysis of the Michaelis–Menten kinetics observed for basal L-proline uptake revealed an apparent K_m of 0.22 ± 0.08 mM and a V_{max} of 0.26 ± 0.03 nmol/mg of protein/min (Fig. 1a). The apparent K_m in our cell studies therefore is almost identical to the one reported for proline influx into oocytes (0.25 mM) expressing the opossum SIT1 protein heterologously. Taken together, the specificity of the proline influx system in OK cells therefore resembles in almost all aspects a SIT1-like phenotype.

When OK cells were exposed for up to 4 h to a medium containing only buffer components and glucose but no amino acids, L-proline uptake remained unchanged (0.063 ± 0.023 vs. 0.047 ± 0.012 pmol/mg of protein/min in control cells), whereas when amino acid deprivation was extended to 8 h, L-proline uptake increased significantly. Eadie–Hofstee transformation of the Michaelis–Menten kinetics obtained under conditions of 8 h amino acid deprivation (Fig. 1b) revealed that influx was now mediated by two components (inset). Whereas the high-affinity system possessed an apparent K_m of 0.12 ± 0.01 mM and a V_{max} of 0.28 ± 0.04 nmol/mg of protein/min and, therefore, seemed unchanged between basal and deprivation conditions (solid lines in inset to Fig. 1b) a low affinity but high capacity system with a K_m of 4.4 ± 0.6 mM and a V_{max} of 10.2 ± 0.6 nmol/mg of protein/min (Fig. 1b) now dominated uptake.

To assess whether the increased proline transport capacity involves de novo synthesis of proteins, we performed uptake studies in cells preexposed to 10 μg/ml actinomycin D or 2 μg/ml cycloheximide under amino acid deprivation conditions. Uptake of proline at low substrate concentration was increased threefold upon amino acid deprivation (0.159 ± 0.023 pmol/mg of protein/min) and this increase was completely blunted when cells were treated with the RNA-synthesis inhibitor (0.060 ± 0.020 pmol/mg of protein/min) or the translation inhibitor (0.056 ± 0.013 pmol/mg of protein/min). Those values were not different from uptake rates in cells grown under standard conditions (0.048 ± 0.014 pmol/mg of protein/min) and provide strong evidence that the enhanced L-proline transport under amino acid deprivation involves de novo biosynthesis of proteins.

Since transport processes rely on a proper energy state needed to maintain the membrane potential, we determined intracellular ATP levels in cells grown under standard

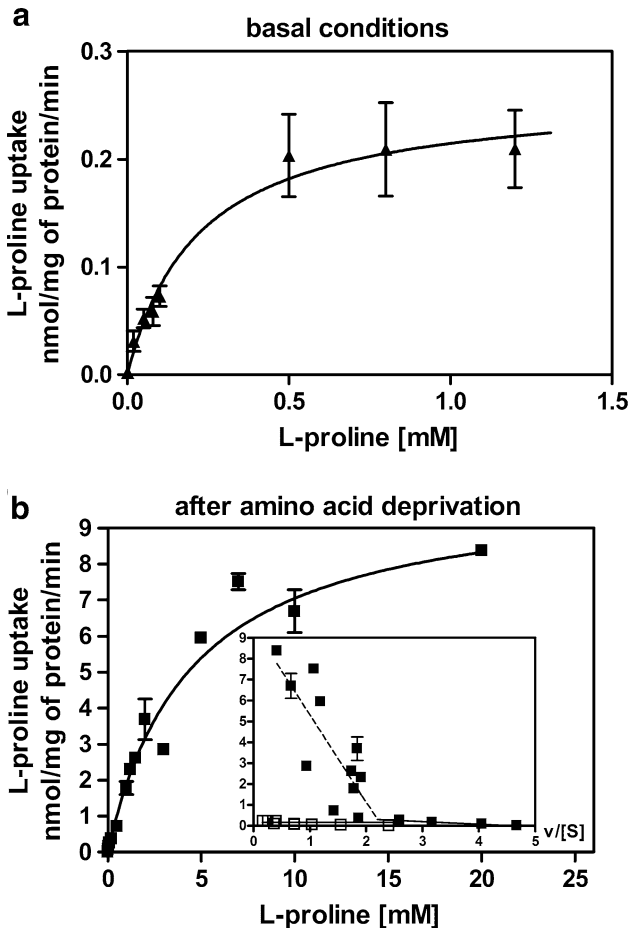

Fig. 1 Kinetics of L-proline uptake under basal conditions and in cells submitted to amino acid deprivation for 8 h. **a** Influx of labeled proline (24 nM [^{3}H]-L-proline) into OK cells grown under standard conditions as determined in the presence of increasing proline concentrations ($n = 6$). The affinity of the transporter was determined by approximation of the Michaelis–Menten type kinetics with an apparent K_m of 0.22 ± 0.08 mM. **b** Kinetics of uptake of proline into OK cells submitted for 8 h to amino acid deprivation as a function of increasing substrate concentrations (0–20 mM; $n = 6$). The Eadie–Hofstee transformation of kinetic data revealed two discrete transport systems (*inset*, *closed squares* as compared to *open squares* for the basal conditions) and approximation of the kinetic constants by a Michaelis–Menten kinetics with two saturable components identified a high affinity–low capacity and a low affinity–high capacity system. Data are mean ± SEM

conditions or submitted for 8 h to amino acid deprivation. However, amino acid deprivation neither significantly altered cellular ATP levels (268 ± 2 µmol/mg protein vs. 254 ± 12 µmol/mg of protein) nor were any signs of apoptotic or necrotic cell death observed. Exposure of cells to 1 mM of AICAR that activates AMP-kinase or to 100 nM of rapamycin that inhibits mTOR (data not shown) also failed to alter proline transport after 8 h of amino acid deprivation and provide a second line of evidence that neither energy status nor mTOR signalling processes are directly involved in the regulation of proline influx.

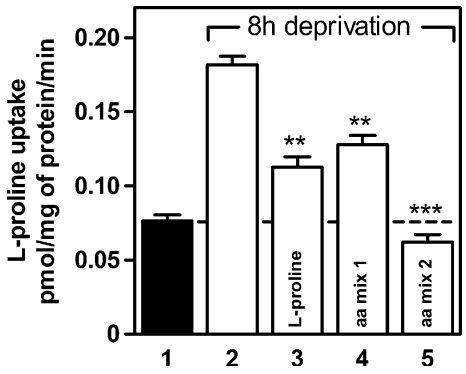

Fig. 2 Modulation of deprivation-induced L-proline uptake by supply of selected amino acids. Tracer proline influx into cells grown under standard conditions or under amino acid deprivation with culture media supplying either 300 µM L-proline (*bar 3*), a mixture (aa mix 1, 750 µM final concentration) of each, 150 µM of Glu, Asp, Arg, His and Lys (*bar 4*) or a mixture (aa mix 2, 900 µM final concentration) of either 150 µM of Ala, Val, Ile, Cys, Ser and Phe (*bar 5*, $n = 3$). Significant differences are denoted as $^{**}p < 0.01$ or $^{***}p < 0.001$. Data are mean ± SEM

For examining whether the adaptation to amino acid deprivation is mediated by a specific proline deficiency or whether other proteinogenic amino acids are involved, we selectively added either L-proline alone in a concentration as found in human plasma (Nasset et al. 1979) or mixtures of selected neutral and charged amino acids, respectively, and reassessed proline influx. When 300 µM L-proline was supplied during the deprivation period, proline uptake rates dropped significantly ($p < 0.001$) as shown in Fig. 2 but were still around 1.5-fold higher than in the absence of proline. When a mixture (aa mix 1) of 150 µM of Glu, Asp, Arg, His and Lys—which are all no SIT1 substrates—was employed, transport still was increased 1.7-fold over that in controls (Fig. 2). In contrast, a mixture of amino acids containing 150 µM of each Ala, Val, Ile, Phe, Cys and Ser (aa mix 2) blunted completely the deprivation-induced upregulation of L-proline influx (Fig. 2). These observations are also characteristic for the expression behavior of SNAT2.

Further characterization of deprivation-induced L-proline influx

In search of candidate transporters that provide the increased L-proline uptake under amino acid deprivation, we investigated the possible contribution of the known renal transporters PAT1 and SNAT2. When Na^+ was replaced by other cations and extracellular pH was lowered to 6.0 providing an optimal driving force for H^+-coupled cotransport of L-proline via PAT1, only low transport rates were obtained (0.014 ± 0.002 pmol/mg of protein/min) and those did not change when cells were amino acid deprived (0.011 ± 0.002 pmol/mg of protein/min). Thus, PAT1 and

PAT2 can be excluded as transporters responsible for the enhanced proline influx observed in OK cells under amino acid deprivation. Whereas a substitution of Na^+ by Li^+ inhibited more than 80% of L-proline uptake in control cells (Table 1) in amino acid-deprived cells uptake still was increased 3.1-fold (to 0.034 ± 0.006 pmol/mg of protein/min) indicating that the high capacity proline transport, seen under deprivation conditions, is Li^+-tolerant.

When substrate specificity was assessed in cells deprived of amino acids employing either tracer proline concentrations or using 1 mM of proline which represents as saturating substrate concentration for the high-affinity system (Fig. 3) major differences in inhibition patterns were observed (Fig. 3a). Uptake rates of tracer proline in the presence of the D-isomers of proline and pipecolate were higher than in control cells indicating that the deprivation-induced high capacity system may not accept these substrates. In contrast, neutral amino acids and histidine but not lysine caused lower proline uptake rates indicating an efficient inhibition in deprived cells.

When a test proline concentration of 1 mM was used, transport was reduced by about 60–70% in the presence of only 1 mM of L-methionine or L-alanine, whereas 10 mM of glycine, L-cysteine, L-serine, and L-histidine were needed to cause similar inhibition rates of 60–80% (Fig. 3b). The D-isomers of proline or pipecolate—that are well accepted as substrates by the high-affinity system—reduced uptake by only 10–15% (Fig. 3b). This substrate selectivity of the transport process in amino acid-deprived cells clearly demonstrated an increased preference for neutral amino acids. We therefore used the radiolabeled amino acids alanine, glycine, and isoleucine in comparison to charged lysine and glutamate to assess the specificity of the adaptation process during deprivation. As shown in Fig. 4, only uptake of proline, alanine and glycine but not that of isoleucine or the charged amino acids increased significantly when amino acid-deprived cells were studied.

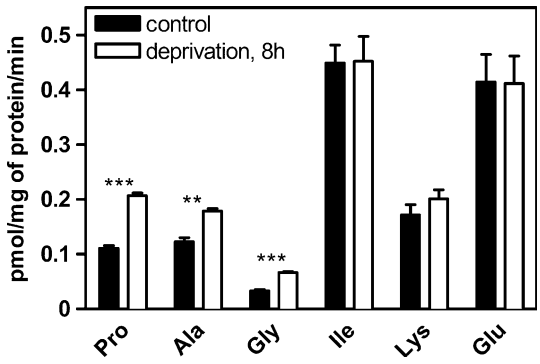

Fig. 4 Transport of selected radiolabelled amino acids under amino acid deprivation. Tracer L-proline (24 nM), L-alanine (21 nM), glycine (48 nM), L-isoleucine (11 nM), L-lysine (10 nM) or L-glutamate (20 nM) influx into cells grown under standard conditions (*black bars*) or for 8 h under amino acid deprivation (*white bars*) ($n = 4$). Significant differences are denoted as $^{**}p < 0.01$ or $^{***}p < 0.001$. Data are mean ± SEM

The observed changes in substrate selectivity with a clear preference for neutral substrates such as alanine, glycine or methionine in combination with the lithium-tolerance seen strongly suggested a SNAT2-like activity mediating the increased proline flux in amino acid-deprived cells. SNAT2 has been described in a variety of tissues (Hatanaka et al. 2000; Mackenzie and Erickson 2004) as a system with these features. We therefore determined apparent mRNA levels of SNAT2 in comparison to those of SIT1 during amino acid deprivation by RT-PCR. After a deprivation period of 4 h, the mRNA level of SIT1 remained unchanged, whereas the transcript level of SNAT2 increased significantly almost threefold (Fig. 5). After 8 h, the transcript levels of SIT1 were increased to 1.6-fold, whereas SNAT2-mRNA decreased slightly from three- to twofold over that in control cells (Fig. 5). This decrease of mRNA levels from 4- to 8-h incubation period could be due to an inhibitory feedback of transcription by the newly synthesized functional proteins.

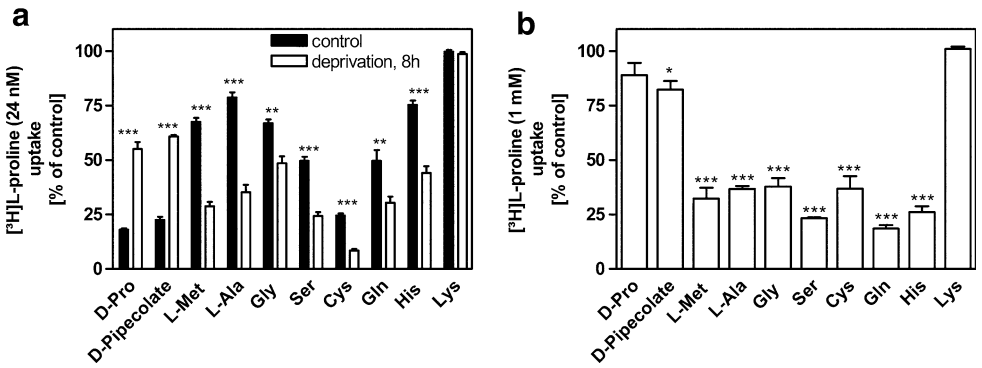

Fig. 3 Substrate specificity of deprivation-induced L-proline uptake. Proline uptake (24 nM **a**; 1 mM **b**) in the absence or the presence of different SIT1 or SNAT2 substrates or lysine provided in concentrations of 1 mM (L-Ala and L-Met) or 10 mM (all other amino acids). Significant differences are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ ($n = 3$–5). Data are mean ± SEM

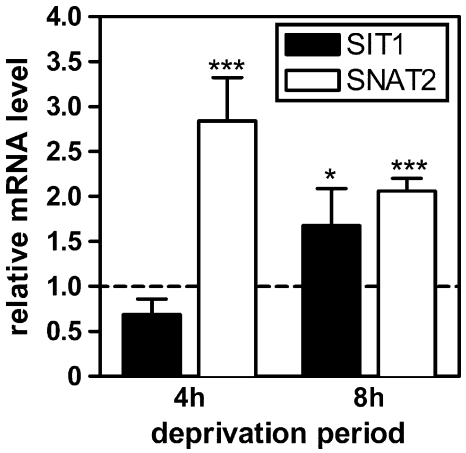

Fig. 5 mRNA levels of SIT1 and SNAT2 genes in cells submitted to amino acid deprivation. mRNA levels were determined by real-time RT-PCR using SYBR Green I with Roche's Light Cycler and are given as relative factor to the amount in non-deprived cells (1.0). ($n = 4$; data represent two independent duplet replications). *Dark columns* mRNA levels of SIT1; $*p < 0.05$ versus non-deprived cells, *light columns* mRNA levels of SNAT2; $***p < 0.001$ versus non-deprived cells

Since SNAT2 activity has been shown to be altered by signalling processes involving JNK, PI-3-kinases and GSK-3, we also determined proline influx after deprivation when cells were simultaneously exposed to inhibitors of these kinases. The inhibitors of JNK (50 μM) or 100 nM of wortmannin-reduced L-proline uptake even below the levels observed in non-deprived cells, whereas 50 μM of the GSK-3 inhibitor SB-415286 reduced L-proline transport to basal levels thus blocking completely the deprivation-induced increase in transport (Fig. 6).

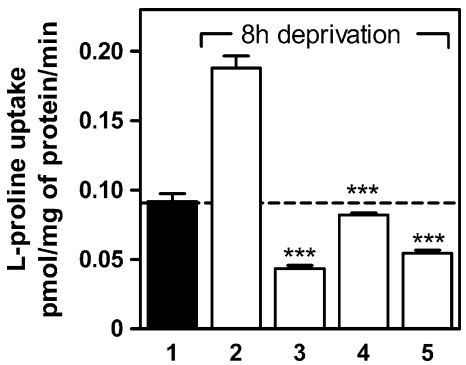

Fig. 6 Effects of selected protein kinase inhibitors on deprivation-induced L-proline uptake. Tracer proline influx into cells grown under standard conditions or for 8 h under amino acid deprivation with culture media supplying in addition 50 μM of JNK inhibitor II (*bar 3*), 50 μM of the GSK inhibitor SB-415286 (*bar 4*) or 100 nM of the PI-3 kinase inhibitor wortmannin (*bar 5*). Significant differences are denoted as $***p < 0.001$ ($n = 3$–5)

Discussion

We here demonstrate for the first time a pronounced upregulation of L-proline influx into renal epithelial cells upon an amino acid deprivation. Whereas proline influx under normal culture conditions is almost exclusively mediated by SIT1 as judged on kinetic behavior, substrate specificity and ion-dependence, amino acid deprivation causes an upregulation of a high capacity system that resembles in all phenotypical aspects SNAT2.

In OK cells cultured under basal conditions L-proline uptake occurs in a Na^+- and Cl^--dependent manner and can be inhibited by all classical SIT1 substrates identified so far. However, in analogy to data provided by Ristic et al. for the OK SIT1 homologue (Ristic et al. 2006) we also demonstrate the ability of some more hydrophobic amino acids to reduce [^{3}H]-L-proline uptake when provided in high concentrations, yet, these amino acids have a much lower affinity than proline. The lack of proline uptake in the absence of Na^+, the lack of a pH effect and the failure of prototypical PAT substrates to inhibit proline flux exclude the possibility of a contribution of PAT1 or PAT2 activity to overall proline uptake.

Additionally, we could exclude B^0AT2 as a candidate transporter since it has a high affinity for methionine, isoleucine and leucine as shown by Bröer et al., whereas we observed only a low-affinity type inhibition by these amino acids (Broer et al. 2006). Moreover, D-proline, which is not a substrate of B^0AT2, efficiently blocked the uptake of L-proline into OK cells. Additionally, cysteine, also not transported by B^0AT2, showed millimolar affinities for the L-proline transport system in OK cells. Taken together, the properties found for proline influx in OK cells with a high affinity for proline, a characteristic ion-dependence as well as the distinct substrate selectivity suggest that SIT1 is the predominant transporter in these cells when grown under standard conditions.

However, when cells were submitted to an amino acid deprivation period of up to 8 h, proline uptake increased several fold and was now dominated by a low affinity but high capacity transport component that was Li^+-sensitive and that could be inhibited by low concentrations of amino acids such as alanine or methionine. The features of this adaptive response resembled a transport mechanism with a SNAT2-like phenotype. The PAT proteins could be excluded because no proton-dependent transport was detectable under deprivation conditions. A contribution of the B^0AT2 systems seems also unlikely as the new high capacity transporter does not cause increased isoleucine uptake which is a substrate of B^0AT2. Moreover cysteine, not transported by B^0AT2, efficiently inhibited the new proline transporting system and lastly this system is Li^+ -tolerant, whereas B^0AT2 is not.

A contribution of the B^0AT1 system, another proline transporter, could also be excluded, since the transport of isoleucine as B^0AT1 substrate was not changed in amino acid-deprived cells and histidine which is a poor substrate for the B^0AT1 system (Bohmer et al. 2005) inhibited proline influx significantly under amino acid deprivation conditions.

SNAT2 is a sodium-coupled neutral amino acid transporter of the *SLC38* family with widespread expression including kidney epithelium. The observed Na^+-dependence but Li^+- tolerance of proline uptake in amino acid-deprived cells is a hallmark of SNAT2 and was also observed in muscle, the hepatoma cell line HepG2, neurons or placental trophoblast cells (Chaudhry et al. 2002; Hatanaka et al. 2000; Jones et al. 2006; Thongsong et al. 2005). SNAT2 was shown to prefer the L-enantiomers of alanine, serine, cysteine, glutamine, asparagine and proline, whereas D-amino acids showed only very low affinity and this resembles our observations regarding the changes in substrate selectivity of the high capacity system as well. SNAT2 is known to be localized in all cell membranes (Hyde et al. 2001, 2002; Ling et al., 2001) including the apical membrane of polarized cells. Since we assessed uptake in polarized cells across the apical side, the high capacity system upregulated under amino acid deprivation conditions seems to correspond well to increased SNAT2-mediated apical uptake in trophoblast cells submitted to amino acid deprivation (Jones et al. 2006; Thongsong et al. 2005).

The increased transport activity in OK cells deprived of amino acids was dependent on de novo protein synthesis as the response could be blocked by inhibitors of protein biosynthesis. The two- to threefold increase in the transcript levels of SNAT2 suggests that increased transcription and translation upon amino acid deprivation leads to the enhanced SNAT2-dependent transport capacity in OK cells. Moreover, the studies with kinase inhibitors and in particular the effect of the GSK-3 inhibitor SB-415286 suggest that in OK cells SNAT2 might be subject to regulation in a similar mode as described for SNAT2 in other cell models (Franchi-Gazzola et al. 1999; Peyrollier et al. 2000). In rat L6 myotubes, SNAT2 protein levels increased threefold and amino acid uptake fourfold after 6-h amino acid deprivation (Hyde et al. 2001) and similarly, in cultured human fibroblasts, a 6-h amino acid deprivation increased transport activity of SNAT2 threefold (Franchi-Gazzola et al. 1999). In these cells as in L6 rat skeletal muscle cells, it was also shown, that increased protein synthesis of SNAT2 generated the transport stimulation (Kashiwagi et al. 2009). Moreover, the deprivation effect was dependent on extracellular regulated kinase 1/2 (ERK) and a cell volume-regulated activation of mitogen-activated protein kinase (MAPK) (Franchi-Gazzola et al. 1999). We also show that the supply of proline alone partially and a mixture of Ala, Val, Ile, Phe, Cys and Ser provided under deprivation conditions completely suppresses the adaptive response and similar findings have been obtained previously. (Boerner and Saier 1985; Kashiwagi et al. 2009).

In summary, we describe here the basic characteristics of SIT1-mediated proline uptake into renal OK cells and the effects of amino acid deprivation on an adaptive increase in proline influx. The latter involves de novo synthesis of new transporters and a change in kinetics of transport as well as substrate specificity that altogether suggests that new SNAT2 transporters now mediate most of proline influx. Amino acid repletion or a GSK-3 inhibitor blunted the deprivation effect on uptake, whereas no evidence for an involvement of AMP-kinase or mTOR was found. Kidney tubular cells therefore seem to possess similar mechanisms as muscle cells or fibroblasts to rapidly alter proline uptake capacity when cells are deprived of amino acids. Our findings demonstrate that a rapid response to changes in renal amino acid handling is provided by upregulation of the SNAT2 transport protein in order to increase the capacity of the renal epithelium for reabsorption and amino acid conservation under fasting and amino acid deprivation conditions.

Acknowledgment This work was supported by the European Framework Programme 6 project EUGINDAT.

References

Bode BP, Kilberg MS (1991) Amino acid-dependent increase in hepatic system N activity is linked to cell swelling. J Biol Chem 266:7376–7381

Boerner P, Saier MH Jr (1985) Hormonal regulation of the System A amino acid transport adaptive response mechanism in a kidney epithelial cell line (MDCK). J Cell Physiol 122:316–322. doi:10.1002/jcp.1041220222

Bohmer C, Broer A, Munzinger M, Kowalczuk S, Rasko JE, Lang F, Broer S (2005) Characterization of mouse amino acid transporter B0AT1 (slc6a19). Biochem J 389:745–751. doi:10.1042/BJ20050083

Boll M, Foltz M, Rubio-Aliaga I, Kottra G, Daniel H (2002) Functional characterization of two novel mammalian electrogenic proton-dependent amino acid cotransporters. J Biol Chem 277:22966–22973. doi:10.1074/jbc.M200374200

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Brandsch M (2006) Transport of L-proline, L-proline-containing peptides and related drugs at mammalian epithelial cell membranes. Amino Acids 31:119–136. doi:10.1007/s00726-006-0307-0

Broer A, Tietze N, Kowalczuk S, Chubb S, Munzinger M, Bak LK, Broer S (2006) The orphan transporter v7–3 (slc6a15) is a Na+-dependent neutral amino acid transporter (B0AT2). Biochem J 393:421–430. doi:10.1042/BJ20051273

Broer S, Bailey CG, Kowalczuk S, Ng C, Vanslambrouck JM, Rodgers H, Auray-Blais C, Cavanaugh JA, Broer A, Rasko JE (2008) Iminoglycinuria and hyperglycinuria are discrete human phenotypes resulting from complex mutations in proline and glycine transporters. J Clin Invest 118:3881–3892. doi:10.1172/JCI36625

Chaudhry FA, Schmitz D, Reimer RJ, Larsson P, Gray AT, Nicoll R, Kavanaugh M, Edwards RH (2002) Glutamine uptake by neurons: interaction of protons with system a transporters. J Neurosci 22:62–72

Franchi-Gazzola R, Visigalli R, Bussolati O, Dall'Asta V, Gazzola GC (1999) Adaptive increase of amino acid transport system A requires ERK1/2 activation. J Biol Chem 274:28922–28928. doi:10.1074/jbc.274.41.28922

Hatanaka T, Huang W, Wang H, Sugawara M, Prasad PD, Leibach FH, Ganapathy V (2000) Primary structure, functional characteristics and tissue expression pattern of human ATA2, a subtype of amino acid transport system A. Biochim Biophys Acta 1467:1–6. doi:10.1016/S0005-2736(00)00252-2

Hyde R, Christie GR, Litherland GJ, Hajduch E, Taylor PM, Hundal HS (2001) Subcellular localization and adaptive up-regulation of the System A (SAT2) amino acid transporter in skeletal-muscle cells and adipocytes. Biochem J 355:563–568

Hyde R, Peyrollier K, Hundal HS (2002) Insulin promotes the cell surface recruitment of the SAT2/ATA2 system A amino acid transporter from an endosomal compartment in skeletal muscle cells. J Biol Chem 277:13628–13634. doi:10.1074/jbc.M108609200

Jones HN, Ashworth CJ, Page KR, McArdle HJ (2006) Expression and adaptive regulation of amino acid transport system A in a placental cell line under amino acid restriction. Reproduction 131:951–960. doi:10.1530/rep.1.00808

Kashiwagi H, Yamazaki K, Takekuma Y, Ganapathy V, Sugawara M (2009) Regulatory mechanisms of SNAT2, an amino acid transporter, in L6 rat skeletal muscle cells by insulin, osmotic shock and amino acid deprivation. Amino Acids 36:219–230

Kennedy DJ, Gatfield KM, Winpenny JP, Ganapathy V, Thwaites DT (2005) Substrate specificity and functional characterisation of the H+/amino acid transporter rat PAT2 (Slc36a2). Br J Pharmacol 144:28–41. doi:10.1038/sj.bjp.0706029

Kowalczuk S, Broer A, Munzinger M, Tietze N, Klingel K, Broer S (2005) Molecular cloning of the mouse IMINO system: an Na+ and Cl^--dependent proline transporter. Biochem J 386:417–422. doi:10.1042/BJ20050100

Ling R, Bridges CC, Sugawara M, Fujita T, Leibach FH, Prasad PD, Ganapathy V (2001) Involvement of transporter recruitment as well as gene expression in the substrate-induced adaptive regulation of amino acid transport system A. Biochim Biophys Acta 1512:15–21. doi:10.1016/S0005-2736(01)00310-8

Mackenzie B, Erickson JD (2004) Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. Pflugers Arch 447:784–795. doi:10.1007/s00424-003-1117-9

Nasset ES, Heald FP, Calloway DH, Margen S, Schneeman P (1979) Amino acids in human blood plasma after single meals of meat, oil, sucrose and whiskey. J Nutr 109:621–630

Peyrollier K, Hajduch E, Blair AS, Hyde R, Hundal HS (2000) L-Leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. Biochem J 350(Pt 2):361–368. doi:10.1042/0264-6021:3500361

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36. doi:10.1093/nar/30.9.e36

Ristic Z, Camargo SM, Romeo E, Bodoy S, Bertran J, Palacin M, Makrides V, Furrer EM, Verrey F (2006) Neutral amino acid transport mediated by ortholog of imino acid transporter SIT1/SLC6A20 in opossum kidney cells. Am J Physiol Renal Physiol 290:F880–F887. doi:10.1152/ajprenal.00319.2005

Takanaga H, Mackenzie B, Suzuki Y, Hediger MA (2005) Identification of mammalian proline transporter SIT1 (SLC6A20) with characteristics of classical system imino. J Biol Chem 280:8974–8984. doi:10.1074/jbc.M413027200

Thongsong B, Subramanian RK, Ganapathy V, Prasad PD (2005) Inhibition of amino acid transport system a by interleukin-1beta in trophoblasts. J Soc Gynecol Invest 12:495–503. doi:10.1016/j.jsgi.2005.06.008