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Role and characteristics of selected amino acid and peptide transporters in epithelial cells

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Zum Erfolg braucht der Forscher die vier großen "G": Geist, Geduld, Geld und Glück.

Paul Ehrlich

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1.1 Introduction

1.1.1 Physiological importance of amino acids

Amino acids contain both amine and carboxyl functional groups, the general formula is $H_2NCHRCOOH$, where R is an organic substituent. R represents a side chain specific to each amino acid. This side chain makes them behave like a weak acid, a weak base, a hydrophile, if they are polar and hydrophobic, if they are nonpolar. Amino acids are essential to life and have a variety of roles in metabolism. Besides their important function as building blocks in proteins, they also form parts of coenzymes, and are precursors for the synthesis of molecules such as heme.

When taken up into the body from nutrition, the 20 proteinogenic amino acids are either used to synthesize proteins and other biomolecules or oxidized water and carbon dioxide as a source of energy (Sakami and Harrington, 1963) with urea as the prime product via nitrogen is excreted. Of the 20 proteinogenic amino acids, 8 (valine, leucine, isoleucine, methionine, threonine, tryptophan, phenylalanine and lysine) are essential for humans and must be obtained from food (Young, 1994). Another group of amino acids is categorised as semi-essential for infants as the metabolic pathways by which these amino acids are synthesized are not fully developed yet and this group comprises cysteine, taurine, tyrosine, histidine and arginine (Imura and Okada, 1998; Lourenco and Camilo, 2002). Furthermore, some amino acids also have non-proteinogenic functions such as tryptophan serving as a precursor of the neurotransmitter serotonin or glutamate that is by itself a neurotransmitter or glycine that serves as a precursor in porphyrin synthesis (Savelieva *et al.*, 2008; Shemin *et al.*, 1948).

1.1.2 Basic principles of amino acid transport

The plasma membrane defines cell size, segregates intra- and extracellular compartments, is crucial for cell-cell communication and the absorption and reabsorption of essential nutrients from the external environment. The exchange with the environment takes place via passive or active mechanisms by transport proteins localized in the hydrophobic domains of the plasma membrane. These proteins regulate in- and efflux of essential compounds like glucose, amino acids, nucleotides, anorganic ions and pharmaceuticals and thus maintain nutrient homeostasis within the cell and whole organism.

In the small intestine, peptides and amino acids are released from dietary proteins during digestion and are efficiently absorbed by the enterocytes of the small intestine (Adibi *et al.*,

1967). Inside the epithelial cells peptides are further hydrolyzed and together with the absorbed free amino acids released into blood circulation and delivered to all tissues. Besides small intestinal cells, kidney epithelial cells also have a high capacity for transport of amino acids since unbound amino acids are filtered in the glomerulum and subsequently reabsorbed into tubular cells to avoid an excessive loss of amino acids via urine.

Inititially transport of nutrients across epithelia was thought to occur via passive diffusion. It is established now that numerous transporter proteins mediate nutrient fluxes and those transporters either couple the uptake of their substrate to the cotransport of Na^+ or H^+ or allow nutrient flux via exchange mechanisms. Na^+-dependent transporters require an inwardly directed Na^+ electrochemical ion gradient, which is maintained by the plasma membrane Na^+/K^+-ATPase. Certain epithelial amino acid transporters have also been reported to be H^+-coupled (Thwaites *et al.*, 1993a) in analogy to brush-border H^+-coupled oligopeptide transporters (Fei *et al.*, 1994). The required inwardly directed H^+ electrochemical ion gradient is maintained by the concerted action of the basolateral Na^+/K^+-ATPase and the brush-border electroneutral Na^+/H^+ exchanger. Concentrative uptake of amino acids is also driven in some cases either by the outwardly directed concentration gradient of other amino acids by exchange mechanisms or simply by the membrane potential.

1.1.3 Amino acid transport in kidney

In the kidney low molecular weight compounds of the blood plasma, like amino acids, are quantitatively filtered in the glomeruli. Assuming a total plasma concentration of 2.5 mM amino acids with a mean molecular weight of 115 and a glomerular filtration rate of 120 ml/min, the amount of amino acids reabsorbed per day in kidney is ~ 50 g (Verrey et al., 2005). More than 95% of the amino acids from the ultrafiltrate are reabsorbed in the S1 – S3 segments of the proximal tubules (Broer et al., 2005), while basolateral transport systems are responsible for their release back into blood circulation. The kidney tubules are lined by an epithelial monolayer and the luminal surface of the cells is covered with densely packed microvilli forming a brush-border membrane. This enormous surface enlargement determines a high apical transport capacity for uptake of amino acids from the luminal side followed by release from the cell back into circulation.

Fig. 1 shows as a cartoon the most important amino acid transporting systems in the apical and basal membranes of kidney tubular cells.

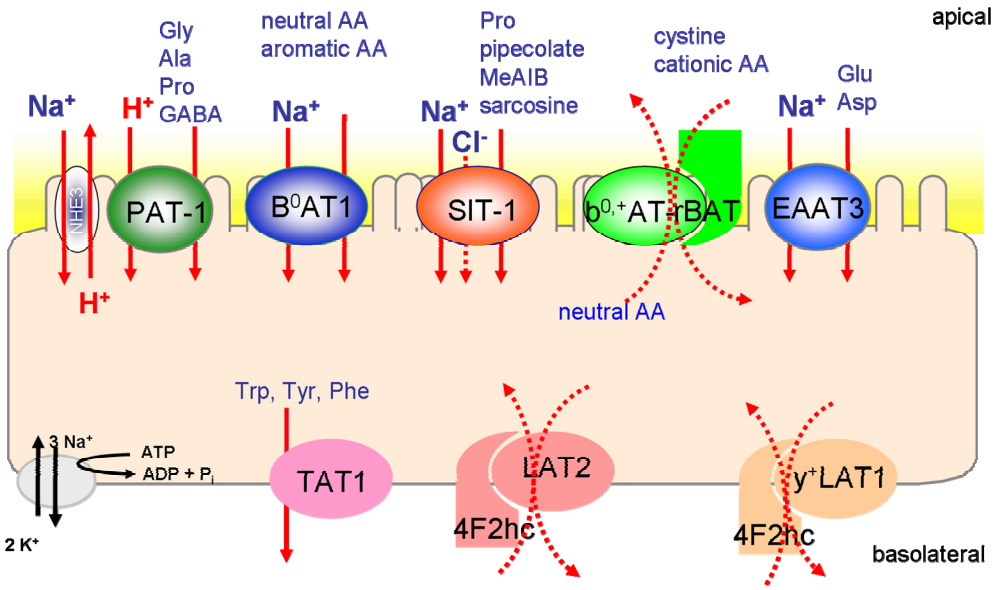

Fig. 1 - Overview of epithelial amino acid transporting systems in kidney

1.1.3.1 Apical amino acid transporters of the kidney proximal tubule

Reabsorption of amino acids in the kidney tubular cells is mostly achieved by Na^+-dependent and uphill transporting mechanisms with four prominent amino acid transport systems contributing to uptake of amino acids from the ultrafiltrate:

- system B^0AT1
- system $b^{0,+}AT$-rBAT
- system EAAT3
- system IMINO

The B^0-type transporter, called B^0AT1 (Slc6a19), belongs to a cluster of orphan transporters within the family of Na^+- and Cl^--dependent neurotransmitter and amino acid transporters (SLC6). B^0AT1 mediates the reabsorption of neutral amino acids in the early segments of the proximal tubule and in epithelial cells of the small intestine. It transports bulk quantities of large neutral amino acids, but also small amino acids like alanine and glutamine. Homozygous carriers of certain mutations in the corresponding gene develop Hartnup disorder (Kleta *et al.*, 2004; Seow *et al.*, 2004), manifested by an increase in the excretion, especially of tryptophan but also of neutral amino-mono- and dicarboxylic acids in urine and usually also in faeces (Baron *et al.*, 1956; Scriver, 1968).

The transport system $b^{0,+}$rBAT mediates a Na^+-independent, high-affinity transport of neutral amino acids, dibasic amino acids and cystine and functions as an exchanger (Van Winkle, 1993) in the S3 segment of the proximal tubule (Furriols *et al.*, 1993). Extracellular cystine or cationic amino acids are preferentially exchanged against neutral amino acids leaving the

cell. These neutral amino acids can be recycled and taken up again into the cell via the B^0-type Na^+ -cotransporter (Mora *et al.*, 1996) allowing sufficient intracellular neutral amino acids to be available for adequate exchange function. The $b^{0,+}$rBAT system is expressed not only in the kidney but also in intestinal epithelia (Magagnin *et al.*, 1992; Stevens *et al.*, 1984). A heterodimeric character of this transporter was suggested after the expression cloning of the type II glycoprotein rBAT in *Xenopus laevis* oocytes (Pfeiffer *et al.*, 1999). The catalytic subunit $b^{0,+}$AT when expressed in *Xenopus laevis* oocytes needs association with the rBAT protein to reach the cell surface membrane (Pfeiffer *et al.*, 1999). Mutations in one of the corresponding genes leads to cystinuria, the most common primary inherited aminoaciduria, which is associated with an excessive urinary excretion of cationic amino acids and cystine.

The anionic amino acids glutamate and aspartate are transported by the high-affinity, Na^+ -dependent symporter EAAT3. Highest levels are found in the later segments S2 and S3 (Shayakul *et al.*, 1997) of the proximal tubule. Besides kidney, EAAT3 is expressed in brain and in small intestine (Kanai and Hediger, 1992). EAAT3 is a strong candidate for the dicarboxylic aminoaciduria, which is generally considered to be a benign disorder, but cases with mental retardation have been reported as well (Swarna *et al.*, 1989; Teijema *et al.*, 1974).

The system IMINO mediates especially the transport of proline. It is described in detail in a later section.

1.1.3.2 Basolateral amino acid transporters of the kidney proximal tubule

Amino acids reabsorbed are released again into blood circulation by basolateral transport systems. However, only a limited number of studies have analyzed the properties of amino acid transport across the basolateral membrane. The well-characterized heterodimeric exchanger y^+LAT1-4F2hc performs the basolateral efflux of cationic amino acids in exchange for aromatic and other large neutral amino acids together with Na^+ (Bauch *et al.*, 2003; Deves *et al.*, 1992; Feliubadalo *et al.*, 1999; Kanai *et al.*, 2000; Pfeiffer *et al.*, 1999) and is highly expressed in the proximal convoluted tubule and the small intestine. Lysinuric protein intolerance is a complex disease that maps to y^+LAT1-4F2hc and presents many symptoms, including the malabsorption and urinary loss of cationic amino acids.

The LAT2-4F2hc transporter functions as an exchanger of neutral amino acids except proline and is highly expressed in the small intestine and proximal convoluted tubule (Rossier *et al.*, 1999; Segawa *et al.*, 1999). Neutral amino acids bind with micromolar affinities to the outside (range 40–200 µM) (Segawa *et al.*, 1999), whereas cytosolic affinities are in the range of 3–30 mM (Meier *et al.*, 2002). Furthermore, LAT2-4F2hc has been shown to be important for

cysteine release and therefore might be a candidate for isolated cystinuria. TAT1 is another basolateral transporter that represents a facilitated diffusion pathway that mediates efflux of neutral and cationic amino acids (Ramadan *et al.*, 2007). In addition, for the anionic amino acids glutamate and aspartate a Na^+ -dependent high-affinity symporter, EAAT2, has been described in the basolateral membrane of renal epithelial cells (Welbourne and Matthews, 1999).

The following Table 1 compiles the major amino acid transporting systems found in mammalian cells with their properties and expression patterns subdivided into Na^+-dependent and Na^+-independent pathways for neutral, cationic and anionic amino acids:

system	example gene	SLC number designation	superfamily designation	transport type coupling ions	amino acid substrates	expression
sodium dependent systems for neutral amino acids						
A	SNAT2	SLC38A2	ATF1	C/ Na^+	A N C Q G H M P S	Ub
ASC	ASCT1	SLC1A4	SDS	A/ Na^+	A S C	K
B^0	B^0AT1	SLC6A19	SDS	C/ Na^+	neutral AA	K,I (AM)
B^0	B^0AT2	SLC6A15	SDS	C/ Na^+	P L V I M	K
Gly	GlyT2	SLC6A9	NTS	C/ Na^+, Cl^-	G	K (AM)
IMINO	SIT1	SLC6A20	---	C/ Na^+, Cl^-	P	K,I (AM)
N	SNAT5	SLC38A5	ATF	C/ Na^+ + E/ H^+	Q N H S (G A)	K,I (BM)
sodium independent systems for neutral amino acids						
asc	Asc1/4F2hc*	SLC7A10	APC + 4F2hc	preferentially E	G A S C T	K,I (AM)
PAT	PAT 1	SLC36A1	APC	C/ H^+	G A P GABA	K,I (AM)
L	LAT1/4F2hc*	SLC7A5	APC + 4F2hc	E	H M L I V F Y W Q	
T	TAT1	SLC16A1	Monocarboxylate	F	W Y F	K,I (BM)
sodium dependent systems for anionic amino acids						
X^{-c}	xCT/4F2hc*	SLC7A11	APC + 4F2hc	E	cystine	Ub
sodium independent systems for anionic amino acids						
X^-_{AG}	EAAT1	SLC1A3	SDS	C/Na^+, H^+, K^+	E D	K (BM)
sodium dependent systems for cationic amino acids						
$B^{0,+}$	ATB(0, +)	SLC6A14	NTS	C/ Na^+, Cl^-	K R A S C T N Q H	I (AM)
y^+L	y^+LAT1/4F2hc*	SLC7A1	APC + 4F2hc	E	K R Q H M L	K,I (BM)
sodium independent systems for cationic amino acids						
$b^{0,+}$	b(0,+)AT/rBAT	SLC7A9	APC + rBAT	E	K R A S C T N Q H	K,I (AM)
y^+	Cat-1	SLC7A1	APC	E (F)	R K H	Ub

Table 1 - Epithelial amino acid transporters and their mediators. K, kidney; I, intestine; AM, apical membrane; BM, basolateral membrane; Ub, ubiquitous. Amino acids are given in one letter codes.

1.1.4 Proline transporting systems

The side chain of proline is a cyclic structure of pyrrolidine, the N-terminus forms an imino group and thus proline is not an amino acid, but an imino acid (Fig. 2).

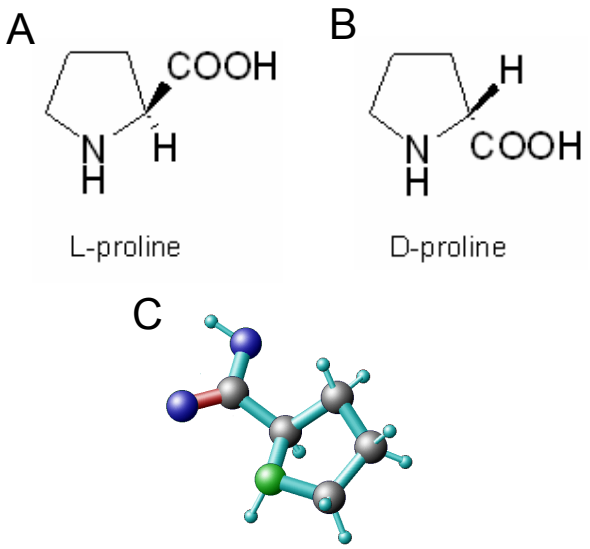

Fig. 2 - Chemical structures of proline (A) and (B); (C) 3D model of the proline structure.

Proline is a major amino acid in collagen proteins. It is hydroxylated in the protein complex and lacks a hydrogen on the α-amino group, so it cannot donate a hydrogen bond to stabilise an α-helix or a β-sheet leading to a kink in the peptide chain. Therefore, proline is responsible for the spacial constitution of the collagen fibres. Unlike other amino acids, which exist almost exclusively in the trans-form in polypeptides, proline exists also in the cis-configuration in peptides. Thus, proline gives a high resistance to most mammalian proteolytic enzymes (Vanhoof *et al.*, 1995).

Proline is not an essential amino acid, as it can be synthesized from glutamate. Concentrations of proline are much lower in plasma than inside mammalian cells (Divino Filho *et al.*, 1997), indicating active transport systems to be involved. However, due to its imino structure it needs specialised transporting systems.

1.1.4.1 Classical system IMINO

The characteristics of transport of L-proline in small intestine, kidney and choroid plexus was already described in the late 1970's (Hammerman and Sacktor, 1977; Munck, 1985; Ross and Wright, 1984) and L-proline was shown to be absorbed completely in the proximal tubule of kidney (Silbernagl *et al.*, 1975) and as shown 1985 by Stevens and Wright by a transport system, which transports exclusively L-proline. This system prefers substrates with an heterocyclic nitrogen-ring, L-stereoisomers and neutral amino acids, especially methylated derivatives. Besides L-proline, the system IMINO prefers L-pipecolate, hydroxyproline, proline

methylester, betaine and methyl-amino isobutyric acid. Cationic amino acids and alanine are not transported. The transport is Na^+-dependent, Cl^- -dependence varies in different species (Munck and Munck, 1997). At physiological Na^+-concentrations the affinity for L-proline is around 0.3 mM (Ross and Wright, 1984; Stevens and Wright, 1985). It is suggested that system IMINO is involved in iminoglycinuria, which is an autosomal-recessive disease with an urinary excretion of proline, hydroxyproline and glycine occurring with an estimated frequency of 1 : 15,000 (Humbertclaude *et al.*, 2001). Normal infants do show frequently a hyperexcretion of these amino acids but when excretion sustains over an age of 6 months, a pathology may be considered. In most cases the disease is benign, blood plasma concentrations are normal and the disease is asymptomatic. Only a few cases with a non-benign phenotype have been described characterized by mental retardation, ichthyosis, neurosensory loss of hearing and convulsions (Joseph *et al.*, 1958; Statter *et al.*, 1976). As some patients showed only an increased excretion of glycine, iminoglycinuria is most likely caused by mutations in more than one gene. When homozygous, all mutations caused renal iminoglycinuria, whereas three variants for heterozygotes could be differentiated in pedigree analysis (Chesney, 2001; Scriver, 1968): (1) Renal iminoglycinuria with an additional impairment of intestinal absorption, heterozygotes are without pathological findings (2) renal iminoglycinuria without an intestinal phenotype, heterozygotes are without pathological findings (3) renal iminoglycinuria without an intestinal phenotype but heterozygotes loose glycine in urine (Kowalczuk *et al.*, 2005). In contrast, an isolated prolinuria was not observed. These observations suggest the involvement of a glycine-specific system and a system shared by proline and hydroxyproline.

Taken together, four transporters are most likely involved in transport of glycine and proline: (1) a common transport system in the intestine and (2) the kidney, (3) a glycine-specific and a (4) proline-specific transport system. Possible candidates are described in detail in following chapters.

1.1.4.2 The proton amino acid transporters PAT 1 and 2

The transport of L-proline was not only observed in the presence of Na^+ but also in the presence of H^+ in the apical membrane of small intestinal cells and rabbit vesicles from pars convoluta of proximal tubule (Roigaard-Petersen *et al.*, 1987; Thwaites *et al.*, 1993b). The first member of the proton-dependent amino acid transporter family was cloned as a lysosomal amino acid transporter 1 (LYAAT1) from rat brain. Subsequently, mPAT1 (SLC36A1, proton-dependent amino acid transporter), the mouse homologues of rLYAAT-1 and mPAT2 (SLC36A2) were cloned from small intestine (Boll *et al.*, 2002; Boll *et al.*, 2004). These proteins consist of about 500 amino acids and are predicted to possess 11 transmembrane do-

mains (Chen *et al.*, 2003; Wreden *et al.*, 2003). Expression studies in oocytes showed the expression in the cell surface, and recently, Broer et al. demonstrated the localization of PAT2 to the proximal tubule brush-border membrane as well (Boll *et al.*, 2002; Broer *et al.*, 2008).

The transport by PAT proteins is energized by an electrochemical H^+ -gradient with proton cotransport with each amino acid by a stoichiometry of 1:1. The H^+ -gradient is established and maintained by the activity of an apical Na^+/H^+ exchanger (Anderson and Thwaites, 2005). The exchanger is driven by an inwardly directed Na^+ gradient, which is established by a Na^+/K^+ -ATPase, localized in the basolateral membrane. In summary, as this ATPase is a primary driving force, the Na^+/H^+ exchanger a secondary-active transport system, the PAT proteins are tertiary active transporters. PAT1 is highly expressed in small intestine, colon, kidney and brain (Boll *et al.*, 2002). PAT2 is mainly found in heart and lung, but also in kidney, testes, liver and spleen. After expression in *Xenopus laevis* oocytes PAT1 was shown to transport small and neutral amino acids such as proline, glycine, alanine, β-alanine, betaine, sarcosine, MeAIB and GABA. The apparent affinities for these substrates are in the range of 3 - 7 mM. PAT1 shows no stereoselectivity and also the D-enantiomers of proline, alanine, serine, cycloserine, pipecolate and hydroxyproline are transported. D-alanine, D-serine, D-cycloserine and D-pipecolate are preferred compared to their L-stereoisomers (Boll *et al.*, 2002; Boll *et al.*, 2004; Kennedy *et al.*, 2005).
PAT2 is in comparison to PAT1 a high-affinity transporter, has a similar substrate specificity with 10 to 30-times higher affinities for the identical substrates but has in general a narrower substrate spectrum. One important difference of the PAT transporters is the pH-dependence: PAT2 shows a higher activity at pH of 6.8 that may exist in kidney than PAT1 (Foltz *et al.*, 2004). Based on the substrate specificity of PAT1, this transporter is a candidate gene for iminoglycinuria associated with an intestinal defect as it is also expressed in the intestine. PAT2 is not expressed in intestine and may be a candidate gene for iminoglycinuria without intestinal phenotype. Although alanine is a substrate of PAT1 and PAT2, its levels are not increased in the urine of iminoglycinuric patients (Scriver, 1989) but this may be explained by the fact that several other renal amino acid transport systems (e.g. system B^0AT1) are known to be involved in alanine reabsorption (Palacin *et al.*, 1998), which may compensate for a defect in the PAT proteins.

1.1.4.3 Transport of L-proline by the Na^+/IMINO acid cotransporter SIT1

As described above, the system PAT1 is a major transport system for uptake of L-proline. However, the classical IMINO carrier was defined as a Na^+ -dependent L-proline transporter, which does not transport L-alanine (Stevens *et al.*, 1982; Stevens and Wright, 1985;

Takanaga *et al.*, 2005b). In 2005, the groups of Hediger and Bröer cloned and characterized the system IMINO from rat, called SIT1 (Sodium/ Imino acid Transporter 1), and from mouse kidney, designated as IMINO^B, respectively. Humans have only one IMINO gene, whereas mouse and rat have two homologues genes corresponding to the single human IMINO gene. In both species, IMINO^B is the active transporter. SIT1/IMINO^B is a member of the Na^+ and Cl^- -dependent neurotransmitter transporter family SLC6 and is encoded by the slc6a20 gene. SIT1 has 12 putative transmembrane domains and two extracellular large loops, each with a predicted *N*-glycosylation site (Fig. 3). rSIT1 is highly expressed in epithelial cells of intestine, kidney tubular S3 segments and choroid plexus (Takanaga *et al.*, 2005b). IMINO^B was mainly found in brain, kidney, small intestine, thymus, spleen and lung (Kowalczuk *et al.*, 2005). IMINO is Na^+ and Cl^- -dependent, transport activity is low at low pH. Replacement of Na^+ by Li^+ reduces uptake of radiolabelled proline completely (Kowalczuk *et al.*, 2005), but small proline-induced currents are still detectable (Takanaga *et al.*, 2005b). High-affinity substrates are L- and D-proline, hydroxyproline, betaine, D- and L-pipecolate with K_m values in a range of 0.1 to 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. Furthermore, IMINO is stereoselective for L-amino acids. Due to its properties, IMINO is also a candidate for iminoglycinuria, although it does not transport glycine. A candidate for defective glycine transport in kidney is the putative glycine transporter XT2. It is found in the plasma membrane, but a transport activity could not be measured. However, an XT2-deficient mouse shows increased levels of glycine in the urine (Quan *et al.*, 2004).

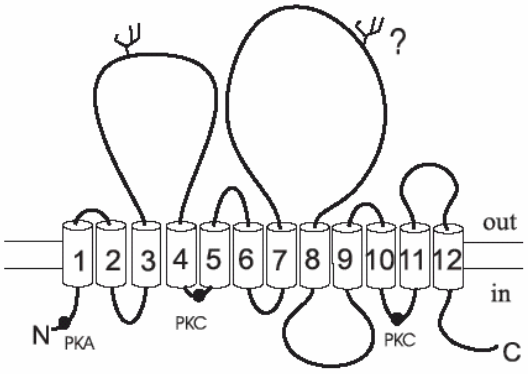

Fig. 3 - Putative secondary structure of SIT1, showing consensus sites for *N*-linked glycosylation, PKA and PKC (Takanaga *et al.*, 2005b)

1.1.4.4 Other proline transporting systems

L-proline is also transported to a minor extent by transport systems for neutral amino acids, such as the system A transporter SNAT2 (previously called ATA2, SA1 and SAT2, SLC38A2) (Takanaga *et al.*, 2002) and the system ASC (SLC1A4). SNAT2 was found in most tissues examined by northern blot analysis, is Na^+ -dependent and is able to transport

alanine, glycine, serine, proline, methionine, asparagine, glutamine, threonine and leucine (Hatanaka *et al.*, 2000). The transporter is sensitive to low pH, highest transport activities are observed at a slightly basic pH. System A is expressed in the blood-brain-barrier and mediates efflux transport of proline from the brain into blood circulation (Takanaga *et al.*, 2002). System ASC transporters are localized in neurons and astrocytes and mediate as Na^+ -dependent neutral amino acid exchangers the transport of proline and hydroxyproline (Pinilla-Tenas *et al.*, 2003; Yamamoto *et al.*, 2003).

In brain, another specific, Na^+ and Cl^- -dependent proline transporter is known and designated as system PROT (Chen *et al.*, 2004). It is not expressed in mammalian epithelia. One more brain-specific proline transporter is well-characterized: SBAT1 (Takanaga *et al.*, 2005a) (SLC6A15). The Na^+ -coupled transporter SBAT1 mediates transport of hydrophobic, zwitterionic α-amino and imino acids with a high affinity for branched-chain amino acids and methionine in a range of 0.08 to 0.16 mM. SLC6A15 was also cloned from mouse by Bröer et al. (Broer *et al.*, 2006). Transport showed a Na^+ -dependence and was Cl^- -independent. Branched-chain amino acids, methionine and proline are preferred substrates. Expression was shown in brain, lung and kidney. However, transport activity could only be detected in synaptosomes and cultured neurons (Broer *et al.*, 2006).

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 localization of the protein in kidney cells was not yet reported. The amino acid transporter B^0AT1, as described in section 1.1.3.1, also transports proline, is localized in the apical membrane of intestinal and renal epithelial cells, but has a very low affinity for proline (Bohmer *et al.*, 2005).

1.1.5 Dietary and adaptive regulation of amino acid transport in kidney

In kidney, the renal reabsorption is highly sensitive to changes in renal amino acid load. For example, taurine is conserved in periods of nutrient deprivation: a reduction of taurine intake leads to a decrease of urinary taurine levels due to its enhanced reabsorption (Chesney *et al.*, 1986; Rozen and Scriver, 1982). A load of taurine however in contrast leads to an increased excretion and these adaptive responses are triggered by changes in the activity of high-affinity taurine transporters (Zelikovic and Chesney, 1989). Another well-characterized mechanism is the adaptive up-regulation of system A activity by amino acid deprivation (Boerner and Saier, 1985) and accordingly, system A is also highly regulated upon alterations of intracellular concentrations of organic osmolytes (Bode and Kilberg, 1991). An amino

acid deprivation was noticeable by an increased expression of the system A transporter gene SNAT2, which lead to an adaptive upregulation of system A in rat skeletal muscle (Hyde *et al.*, 2001), in fibroblasts (Gazzola *et al.*, 2001) and in liver (Varoqui and Erickson, 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). In muscle cells, a 4-fold increased activity and a 3-fold increased protein expression of SNAT2 was detectable upon starvation. In fibroblasts, system A activity was 3-fold increased after a 6 h-period of amino acid deprivation dependent on a 4-fold stimulation of extracellular regulated kinase 1/2 (ERK) and a cell volume-regulated activation of mitogen-activated protein kinase (MAPK) activity (Franchi-Gazzola *et al.*, 1999). System A activity increased by approximately 300% within 7 hours after subconfluent renal MDCK cells were transferred to an amino acid-free medium and this was shown to alter both the K_m and the V_{max} for MeAIB transport and was found to rely on protein synthesis. A readdition of amino acids to the culture medium could repress the increase (Boerner and Saier, 1985). Similarly, in the renal epithelial cell line NBL-1, increased aspartate transport was observed after a 24 h-period of amino acid deprivation with the V_{max} doubled but K_m unchanged. Neither mRNA nor protein levels of EAAT3 (Ferrer-Martinez *et al.*, 1995) were altered (Nicholson and McGivan, 1996) and it was thus suggested that a post-transcriptional regulation with induction of a putative protein activating EAAT3 by low intracellular glutamate levels takes place.

1.1.6 Aim of this work

OK cells are a permanent cell line established from kidney tissue of an adult american opossum (Koyama *et al.*, 1978). They are epithelial-like with a stable nondiploid chromosomal modal number of 23 and are often used as a physiological model system of renal proximal tubular cells (Koyama *et al.*, 1978). This cell line has also been instrumental in characterizing the function of several amino acid transporters, in particular $b^{0,+}$rBAT, LAT2-4F2hc, and B^0AT1 (Fernandez *et al.*, 2003; Mora *et al.*, 1996). Furthermore, SIT1 was also shown to be expressed in OK cells by the group of Verrey (Ristic *et al.*, 2006). Heterologous expression of oSIT1 in oocytes exhibited a high affinity for L-proline exceeding that of isoleucine almost ten-fold, whereas maximal velocity was 3-fold 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.*, 2005b).

Although some OK cell amino acid transporters have been identified, knowledge on regulation of L-proline transport was sparse and therefore the present project attempted

- a) to characterize OK cell proline transport with respect to driving forces and substrate specificities in competition studies with [^{3}H]L-proline and

- b) to assess the regulation of proline transport during amino acid deprivation and possible underlying signalling processes.

1.2 Results

1.2.1 Detection of proline transporter transcripts by PCR in OK cells

PCR was used to assess which proline transporting proteins/genes are expressed in OK cells. Fig. 4A shows the position of primer pairs for SIT1. As Fig. 4B and C show, mRNA of the slc6a20 gene could be detected with all four primer pairs. β-actin served as positive control and its amplicon of 385 bp could also be generated (Fig. 4D). Besides, mRNA of PAT1 and PAT2 could also be amplified by PCR analysis with every two gene specific primer pairs (Fig. 5).

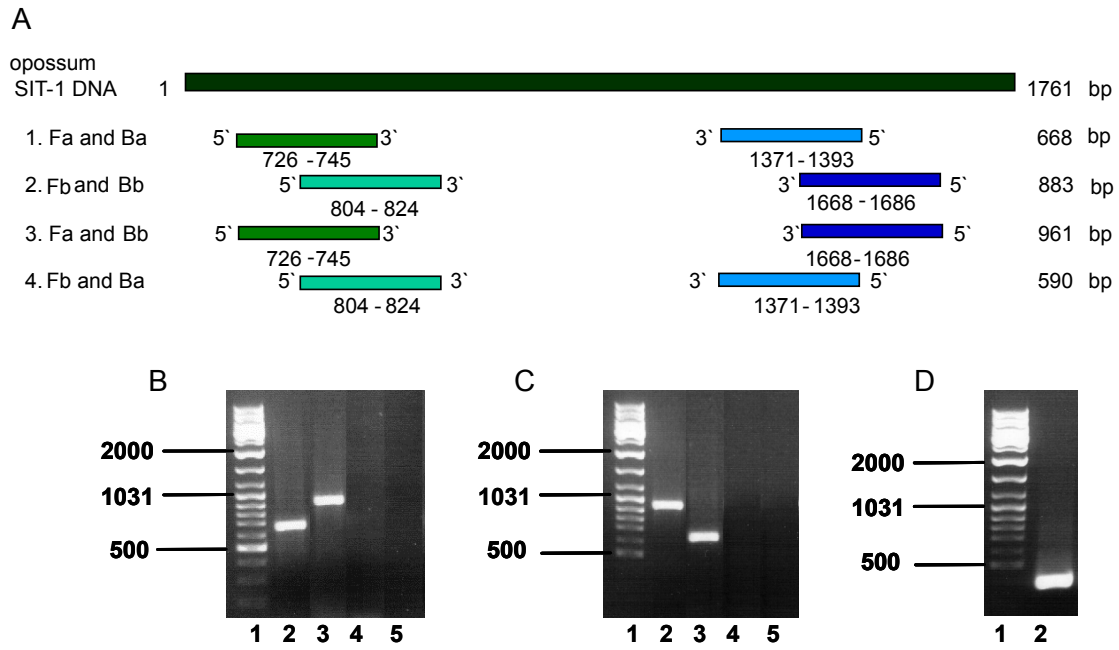

Fig. 4 - Detection of slc6a20 transcript by PCR. (A) Position and possible pairing of opossum SIT1 primer pairs. PCR-fragments amplified from cDNA with gene specific primer pairs. (B) Lane 1: DNA-ladder; lane 2: pp1, 650-700 bp; lane 3: pp2, 900 bp; lane 4 and 5: water controls of pp1 and pp2. (C) Lane 1: DNA-ladder; lane 2: pp3 900-1000 bp; lane 3: pp4, 600 bp; lane 4 and 5: water controls of pp3 and pp4. (D) Lane 1: DNA-ladder; lane 2: control ß-actin primer pair

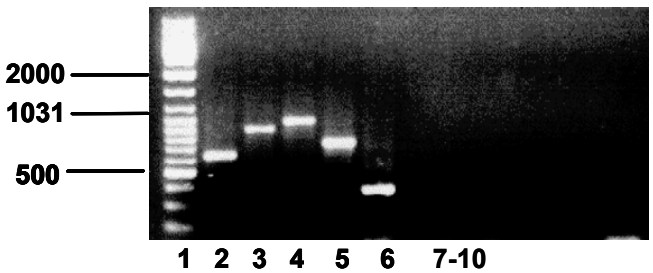

Fig. 5 - Detection of slc36a1 and slc36a2 transcripts by PCR. PCR-fragments amplified from cDNA with gene specific primer pairs. Lane 1: DNA-ladder; lane 2: pat1-pp1, 630 bp; lane 3: pat1-pp2, 900 bp; lane 4: pat2-pp1: Fa/Ba; lane 5: pat2-pp2: Fb/Bb; lane 6: control ß-actin primer pair; lane 7-10: water controls of all primer pairs

So, most obviously all three transporters capable of transporting L-proline are expressed in OK cells.

1.2.2 Time-dependence of [³H]L-proline uptake in OK cells

To determine the linearity of [³H]L-proline uptake, OK cells were incubated with radiolabelled proline for up to 15 min. As Fig. 6 shows, uptake of radiolabelled proline was linear only for up to 5 to 6 min. Due to a better handling of the 6-well-plates, an incubation time of 6 min was chosen.

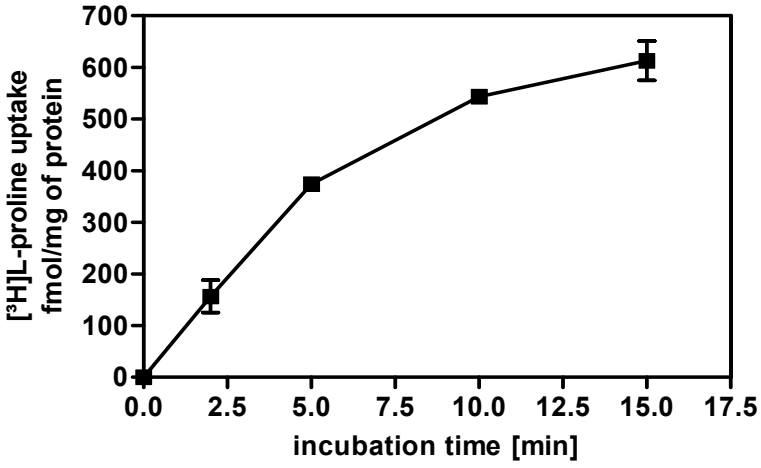

Fig. 6 - Time-dependence of [³H]L-proline uptake in OK cells (n = 3).

1.2.3 Transport kinetics of L-proline uptake in OK cells

Next, functional experiments were performed to characterize L-proline transport in OK cells. The transport kinetics and affinities for L-proline differ markedly between SIT and PAT proteins. SIT1 is a high affinity transporter with a K_m for proline of about 200 µM while the PAT1 protein shows ten times reduced affinity (K_m ~ 2.8 mM). The PAT2 protein shows a higher affinity (K_m ~ 0,12 mM). Here the K_m of the proline flux into OK cells was determined by incubating the cells in media with increasing proline concentration. Half maximal transport rates were derived from approximations of the kinetics according to Michaelis-Menten with an apparent K_m for proline of 0.22 ± 0.08 mM and a V_{max} = 0.26 ± 0.03 nmol/mg of protein/min (Fig. 7). The apparent K_m found here in OK cells is thus almost identical to that reported for proline influx into oocytes (0.25 mM) expressing the opossum SIT1 protein heterologously.

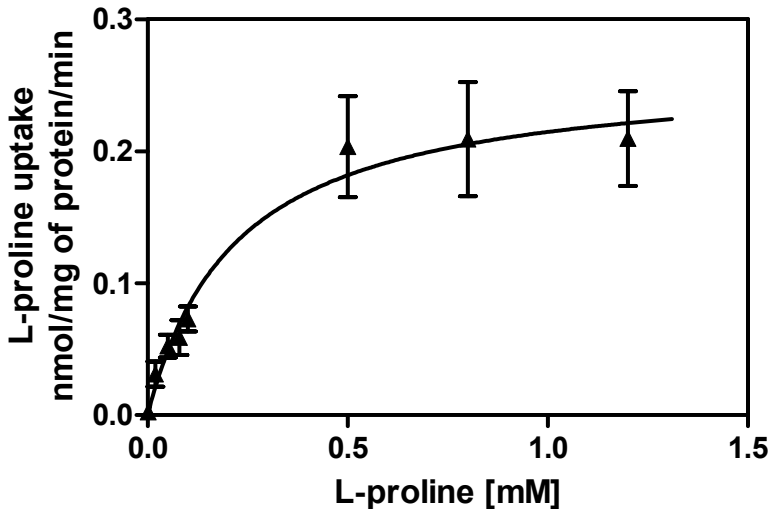

Fig. 7 - Kinetics of L-proline uptake in OK cells. Uptake rates of proline were measured in OK cells in buffer pH 7.4 for 6 min with increasing substrate concentrations consisting of 0 to 1.2 mM unlabelled proline added to 24 nM [³H]L-proline (n = 6).

1.2.4 Determination of ion-dependence of L-proline transport

For further characterization of [^{3}H]L-proline flux, the requirements of Na^+,Li^+,Cl^- and H^+ as cotransport-ions were studied (Fig. 8). The replacement of Na^+ by choline diminishes the transport of proline in the OK cells by 87%, indicating a Na^+-dependent transport mode (Fig. 8A). A substitution of Na^+ by Li^+ reduced [^{3}H]L-proline uptake by about 80% (Fig. 8A) and reducing the chloride concentration from 151 mM to 11 mM or 0 mM reduced proline transport to 54% and 15%, respectively.

When pH in the incubation medium was lowered from 7.4 to 6.5 or 6.0, uptake was reduced by 36% and 42%, respectively (Fig. 8C). In the absence of sodium, no proline uptake was observed at pH 6.0 and pH 7.4, as a proline concentration of 10 mM could not significantly reduce uptake of radiolabelled proline (Fig. 8D). Taken together, L-proline transport in OK monolayers was strongly Na^+ and Cl^- -dependent, Li^+ could only partly substitute for Na^+. The transport was pH-sensitive but could not be driven by H^+ suggesting that PAT proteins are not involved on overall proline uptake into OK cells.

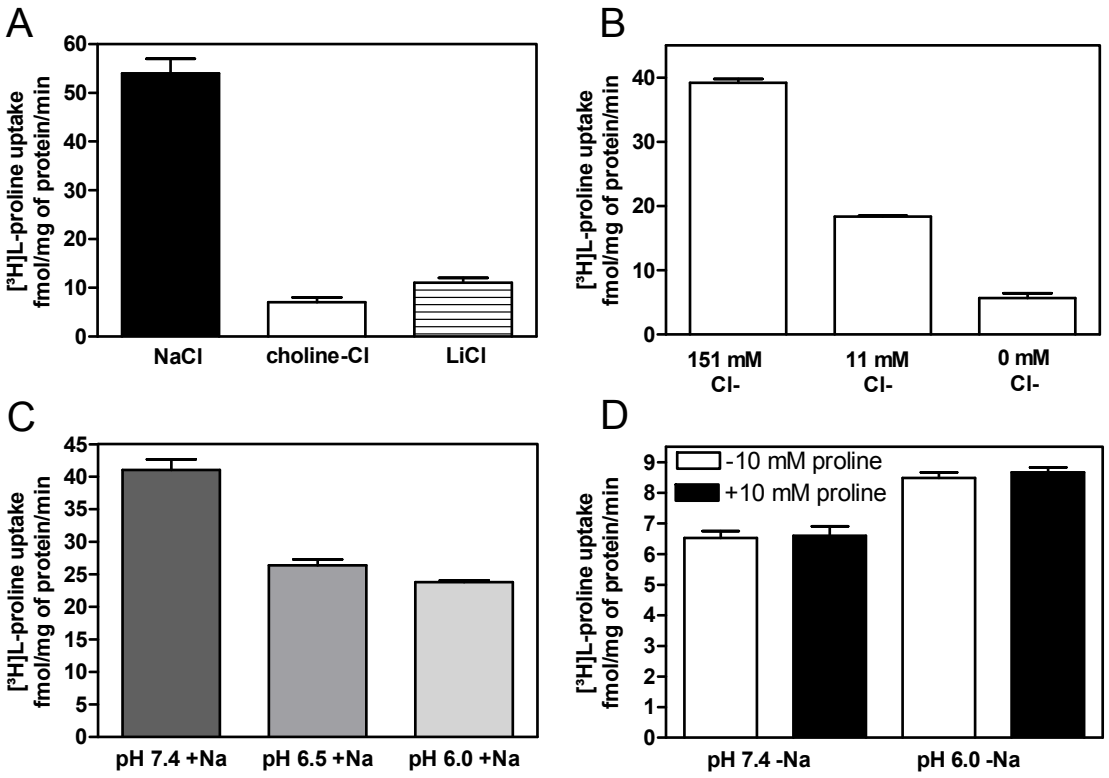

Fig. 8 - Ion-dependence of L-proline transport in OK cells. (A) Na^+-and Li^+-dependence of [³H]L-proline transport. (B) Cl^--dependence of [³H]L-proline transport. (C) pH-dependence of [³H]L-proline transport. (D) H^+-dependence of [³H]L-proline transport. Uptake of [³H]L-proline transport was determined in the absence of Na^+ and at pH-values of 7.4 and 6.0 (n = 4).

To assess the selectivity and specificity of the OK cell proline transport, influx in the absence and the presence of prototypical amino acids for SIT1, B^0AT1, B^0AT2 and the PAT systems was measured.

1.2.5 Specificity of L-proline transport for proteinogenic amino acids and ornithine

All proteinogenic L-amino acids and some D-amino acids were analysed for their ability to reduce [^{3}H]L-proline uptake into OK cells (Fig. 9A). All amino acids were used at a concentration of 10 mM while some amino acids were additionally tested at lower concentrations to estimate their affinity. The amino acids and typical SIT1-substrates L-proline and L-hydroxyproline reduced the uptake of [^{3}H]L-proline nearly completely, whereas the corresponding D-imino acids showed a lower inhibition rate. The other hydrophobic amino acids phenylalanine, valine, leucine, isoleucine and methionine that are substrates typical for B^0AT1 and B^0AT2 also caused inhibition of [^{3}H]L-proline uptake at 10 mM by 65% to 85%. To estimate their affinity, the potential of these hydrophobic amino acids to interact with [^{3}H]L-proline uptake was investigated at a lower concentration of 1 mM. Inhibition varied from 14% to 33% and therefore these hydrophobic amino acids and glutamine may be considered low-affinity type substrates. All other neutral amino acids showed inhibition ≤ 50%, indicating af-

finities ≥10 mM. All typical PAT1/2 substrates like alanine (L- and D-), glycine and D-serine are poor substrates of the proline transporting system in OK cells. Amino acids with charged side chains did not interact with [^{3}H]L-proline (Fig. 9B) except for L-glutamate with a 50% inhibition at high concentrations of 10 mM.

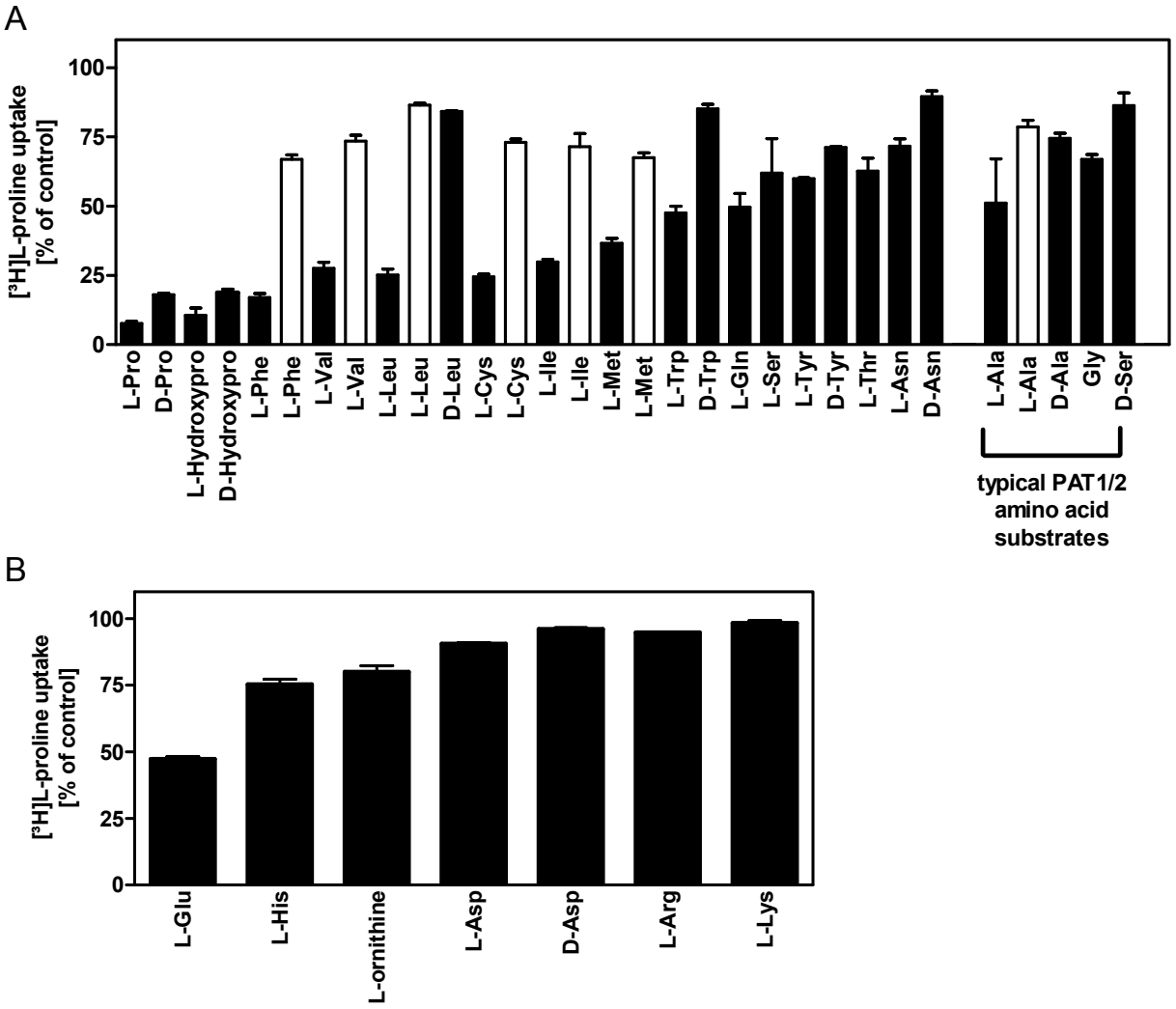

Fig. 9 - Substrate specificity of [³H]L-proline transport for amino acids. (A) Substrate specificity for neutral amino acids. Uptake of [³H]L-proline was determined in the presence of competitors at 10 mM final concentration (black bars), additionally L-Phe, L-Val, L-Leu, L-Cys, L-Ile, L-Met and L-Ala at 1 mM (white bars). (B) Substrate specificity for amino acids with charged side chains at 10 mM final concentration (n = 3-4).

1.2.6 Specificity of L-proline transport for typical SIT1 and PAT1/2 substrates

To further investigate the substrate scope of the proline transport system, OK cells were incubated with [^{3}H]L-proline in the presence of typical SIT1 and PAT substrates at 10 mM (Fig. 10). Pipecolate (L- and D-), N-methylproline, sarcosine, α-N-MeAIB, betaine, N,N-dimethylglycine and (iso)nipecotic acid showed a distinct inhibition and caused transport inhibition by up to 90%. When typical PAT1/2 substrates such as β-alanine and cycloserine (L- and D-) and typical PAT1 substrates such as taurine, vigabatrin and GABA were employed

as competitors, L-proline transport was not significantly affected (Fig. 10). The ability of α-ABA and other N-methylated amino acids to reduce [^{3}H]L-proline transport was further analyzed. α-ABA (10 mM) was able to reduce L-proline uptake dramatically by 80% like N-methylleucine (5 mM) and N-methylalanine (5 mM). Whereas methylation seems to increase affinity of the selected amino acids, N-acetylcysteine showed only a low inhibition of the proline uptake system in OK cells.

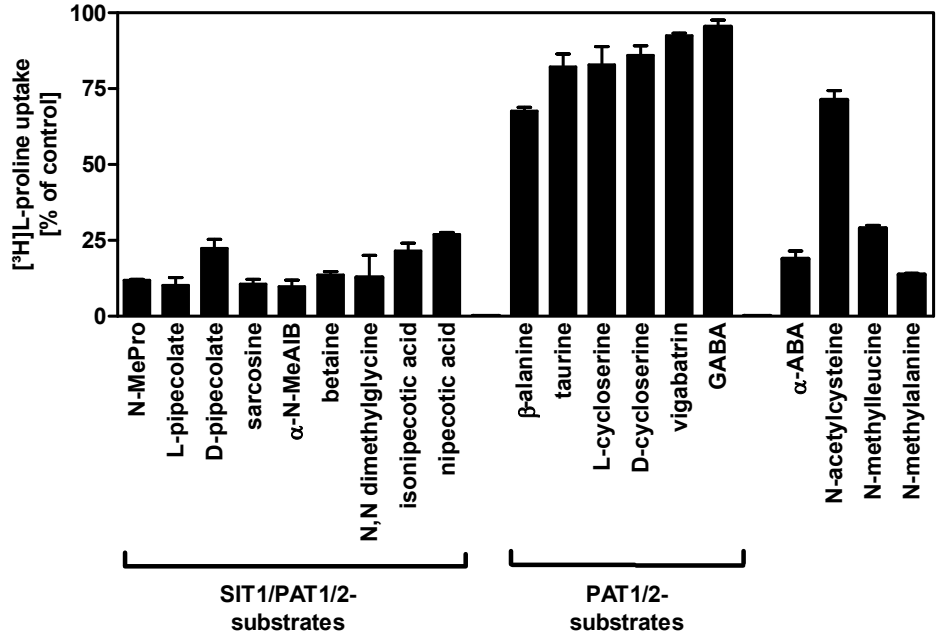

Fig. 10 - Substrate specificity of [³H]L-proline transport for SIT1/PAT1/2 substrates, selected N-methyl amino acids and N-acetylcysteine. Uptake of [³H]L-proline was determined in the presence of competitors (vigabatrin, N-methylleucine and N-methylalanine at 5 mM concentration, the other tested substrates at 10 mM concentration) (n = 3-4).

Taken together, the specificity of the proline influx system in OK cells therefore resembles in almost all aspects a SIT1-like phenotype.

1.2.7 Involvement of the L-proline transport system in the uptake of neutral amino acids

For SIT1 it is postulated that it not only mediates high-affinity reabsorption of L-proline, but also a bulk uptake of other neutral amino acids (Ristic *et al.*, 2006). In OK cells, this possibility was tested by analyzing the potential of L-proline to reduce the uptake of the neutral amino acid alanine (Fig. 11) as a model substrate. Neither at low nor at high alanine concentrations a significant inhibition by proline could be observed (Fig. 11B) indicating that the proline uptake system in OK cells plays only a negligible role in the uptake of neutral amino acids such as alanine.

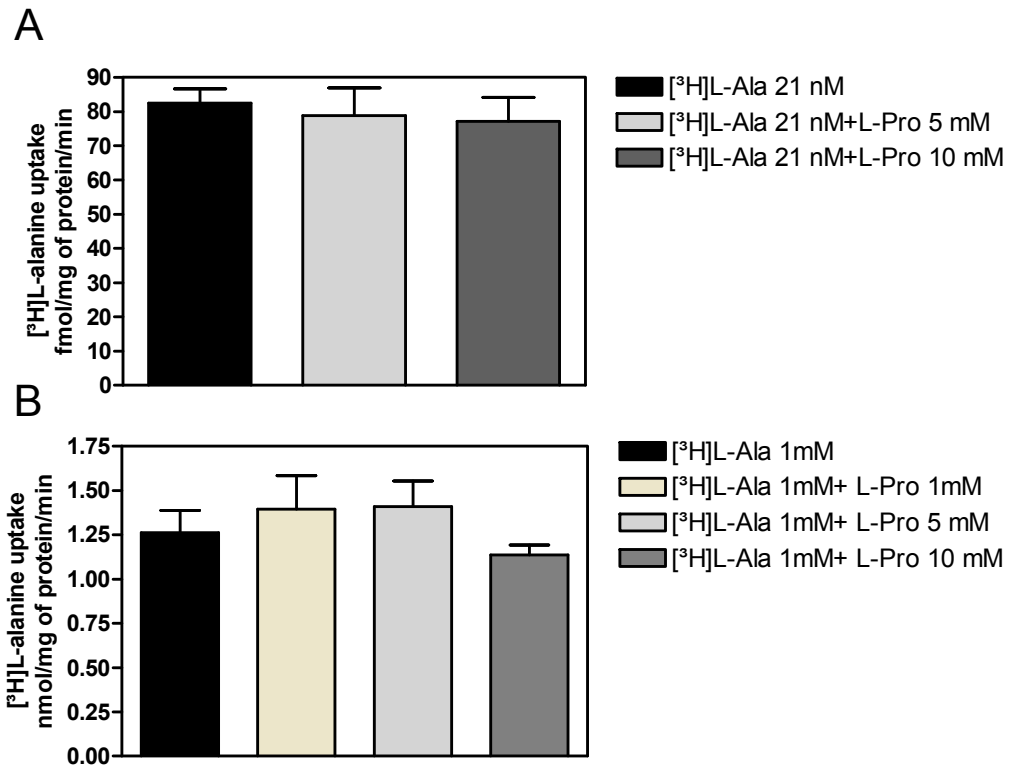

Fig. 11 - Inhibition of [³H]L-alanine uptake by non-labelled L-proline. Uptake of [³H]L-alanine (21nM (A) or 1mM (B), final concentration) was determined in the presence of different concentrations of non-labelled proline (n = 4-6).

1.2.8 Regulation of L-proline transport under amino acid deprivation

To assess whether and how OK cells can regulate proline transport under conditions of different quantities of amino acids available, cells were exposed to amino acid deprivation.

1.2.8.1 Alterations of kinetics of L-proline transport

When OK cells were exposed for up to 4h to a medium containing only buffer components and glucose but no amino acids, L-proline uptake remained unchanged (63 ± 23 vs. 47 ± 12 fmol/mg of protein/min in control cells), whereas when amino acid deprivation was extended to 8h, L-proline uptake increased significantly. Eadie-Hofstee transformation of the Michaelis-Menten kinetics obtained (Fig. 12) revealed that influx was now mediated by two components (insert). 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 insert to Fig. 12), 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. 12) now dominated the uptake.

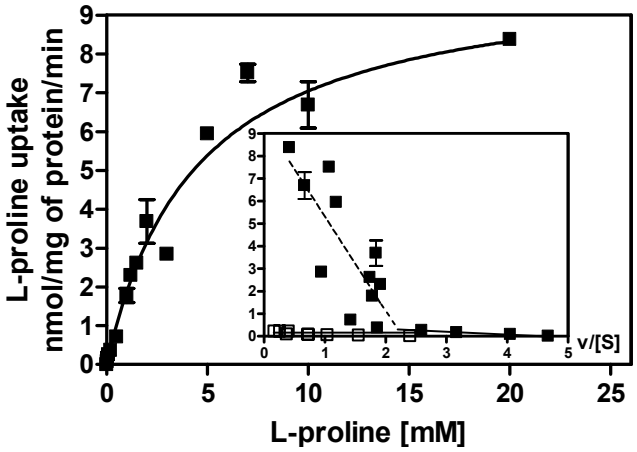

Fig. 12 - Alterations of kinetics of L-proline transport. The Eadie-Hofstee transformation of the kinetic data revealed two discrete transport systems (inset, closed squares as compared to open squares for the basal conditions) and approximation of the kinetic constants by a Michaelis-Menten kinetics with two saturable components identified a high-affinity – low-capacity and a low-affinity – high-capacity system (n = 6).

1.2.8.2 Involvement of protein synthesis

To assess whether the increased proline transport capacity involves *de novo* synthesis of proteins, uptake studies in cells preexposed to 10 µg/ml actinomycin D or 2 µg/ml cycloheximide were performed under amino acid deprivation conditions. Uptake of proline at low substrate concentration was increased 3-fold upon amino acid deprivation of 8h (159 ± 23 fmol/mg of protein/min; Fig. 13) and this increase was completely blunted when cells were treated with the RNA-synthesis inhibitor (60 ± 20 fmol/mg of protein/min) or the translation inhibitor (56 ± 13 fmol/mg of protein/min) and those values were not different to uptake rates in cells grown under standard conditions. These results provide strong evidence that the enhanced L-proline transport under amino acid deprivation involves *de novo* biosynthesis of proteins.

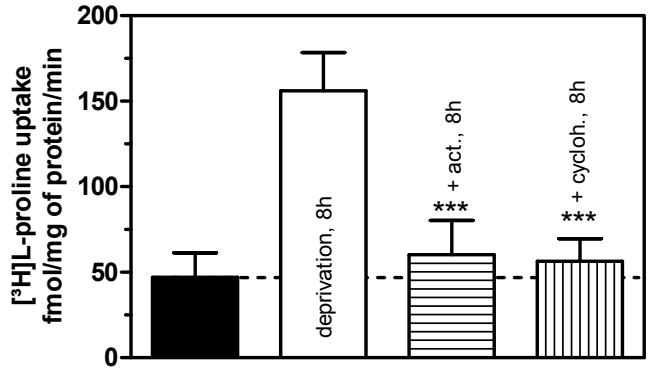

Fig. 13 - Involvement of protein synthesis in increased proline transport. Tracer proline influx into cells grown under standard conditions or under amino acid deprivation for 8h in the absence or presence of actinomycin D (10 µg/ml) or cycloheximide (2 µg/ml) (8h; n = 8); ***p < 0.001 versus amino acid deprived cells.

1.2.8.3 Involvement of energy-sensing mechanisms

Since transport processes rely on a proper energy state needed to maintain the membrane potential, intracellular ATP levels in cells grown under standard conditions or submitted for 8h to amino acid deprivation were determined (Fig. 14A). However, amino acid deprivation did not significantly alter ATP levels (268 ± 2 µmol/mg of protein versus 254 ± 12 µmol/mg of protein) nor were any indication of apoptotic or necrotic cell death under amino acid deprivation conditions observed.

Exposure of cells to 1 mM of AICAR that activates AMP-kinase or to 100 nM rapamycin that inhibits mTOR also failed to alter proline transport after 8h of amino acid deprivation (Fig. 14B) and provide a second line of evidence that neither energy status nor mTOR signalling processes are directly involved in the upregulation phenomenon.

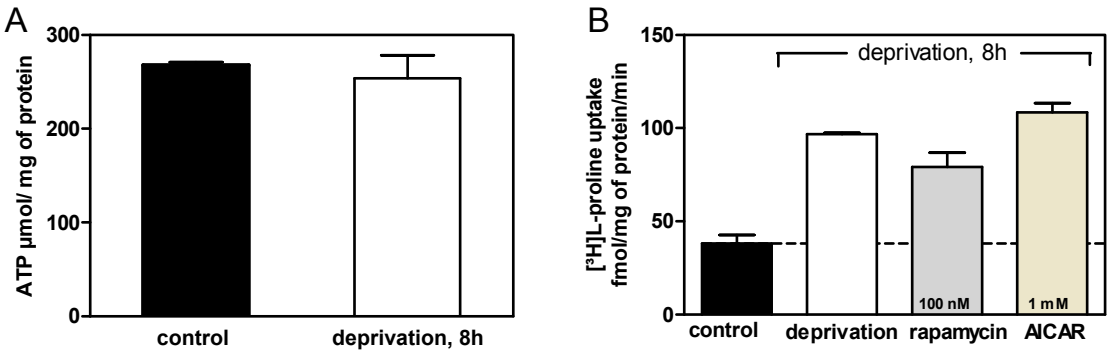

Fig. 14 - Involvement of energy-sensing mechanisms. (A) ATP-levels in control cells compared to amino acid deprived cells. (B) Tracer proline influx into cells grown under standard conditions or under amino acid deprivation for 8h in the absence or presence of rapamycin and AICAR (n = 3).

1.2.8.4 Modulation of increased proline transport by external amino acids

For examining whether the adaptation to amino acid deprivation is mediated by a specific proline deficiency or whether other proteinogenic amino acids are involved, 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 were added and proline influx was determined again. When 300 µM L-proline was supplied during the deprivation period, proline uptake rates dropped significantly ($p<0.001$) as shown in Fig. 15 but were still around 1.5-fold higher than in the absence of proline (Fig. 15). 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 was still increased 1.7-fold over that in controls (Fig. 15). 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. 15).

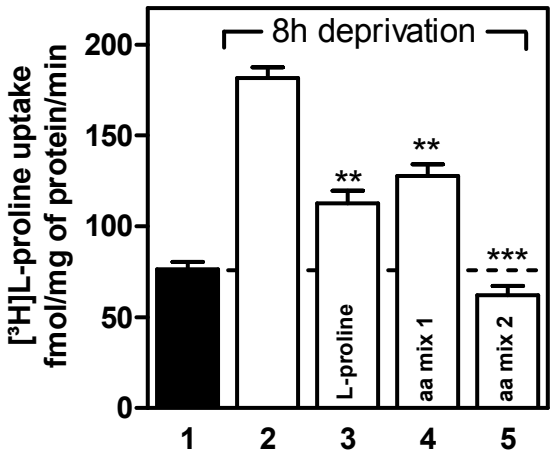

Fig. 15 - Modulation of increased proline transport by amino acids. Tracer proline influx into cells grown under standard conditions or under amino acid deprivation for 8h in the absence or presence of 300 µM L-proline (bar 3), a mixture (aa mix 1, 750 µM total concentration) of each, 150 µM of Glu, Asp, Arg, His and Lys, (bar 4) or a mixture (aa mix 2, 900 µM total 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 *** $p < 0.001$.

1.2.8.5 Changes of ion-dependence

In search of candidate transporters that provide the increased L-proline uptake under amino acid deprivation, the possible contribution of the known renal transporters PAT1 and SNAT2 was investigated. 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 (14 ± 2 fmol/mg of protein/min) and those did not change when cells were amino acid deprived (11 ± 2 fmol/mg of protein/min) (Fig. 16A). Thus, PAT1 and PAT2 could be excluded as transporters responsible for the enhanced proline influx observed in OK cells under amino acid deprivation. A substitution of Na^+ by Li^+ inhibited L-proline uptake by more than 80% in control cells (Fig. 16B), whereas in amino acid deprived cells uptake was still increased 3.1-fold (to 34 ± 6 fmol/mg of protein/min), indicating that the high-capacity proline transport, seen under deprivation conditions, is Li^+-tolerant.

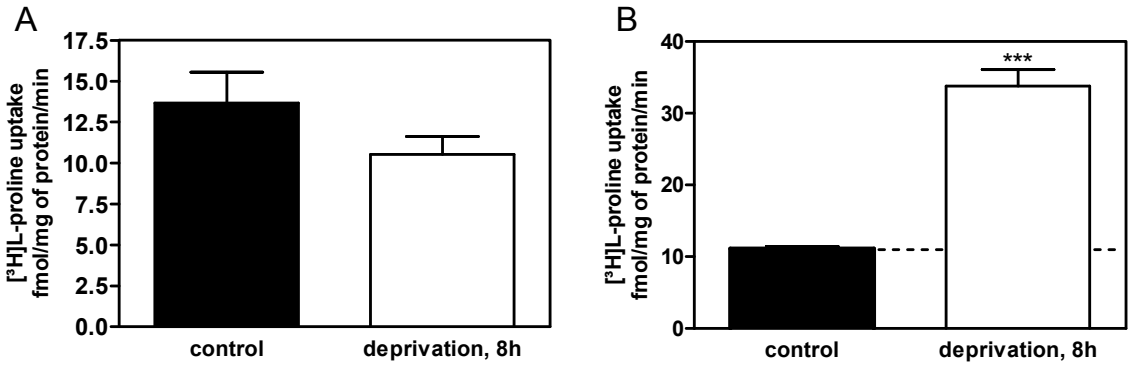

Fig. 16 - H^+- and Li^+- .dependence under amino acid deprivation conditions. (A) Tracer proline influx into cells grown under standard conditions or under amino acid deprivation for 8h at pH of 6.0 in the absence of Na^+ (B) Tracer proline influx into cells grown under standard conditions or under amino

acid deprivation for 8h, Na^+ is replaced by Li^+. Significant differences are denoted as *** $p < 0.001$ (n = 3 to 5).

1.2.8.6 Changes of substrate specificity

When substrate specificity was assessed in cells deprived of amino acids employing either tracer proline concentrations or using 1 mM of proline, which represents a saturating substrate concentration for the high-affinity system, major differences in inhibition patterns were observed. 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 to 70% in the presence of only 1 mM of L-methionine or L-alanine, whereas 10 mM of glycine, L-cysteine, L-serine, L-histidine were needed to cause similar inhibition rates of 60 to 80% (Fig. 17B). Even 10 mM of L-lysine failed to inhibit proline influx and the D-isomers of proline or pipecolate – that are well accepted as substrates by the high-affinity system – reduced uptake by only 10 to 15% (Fig. 17B). This substrate selectivity of the transport process in amino acid deprived cells clearly demonstrated an increased preference for neutral amino acids. Therefore, the radiolabelled amino acids alanine, glycine, and isoleucine were used in comparison to charged lysine and glutamate to assess the specificity of the adaptation process during deprivation. As shown in Fig. 17C, 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.

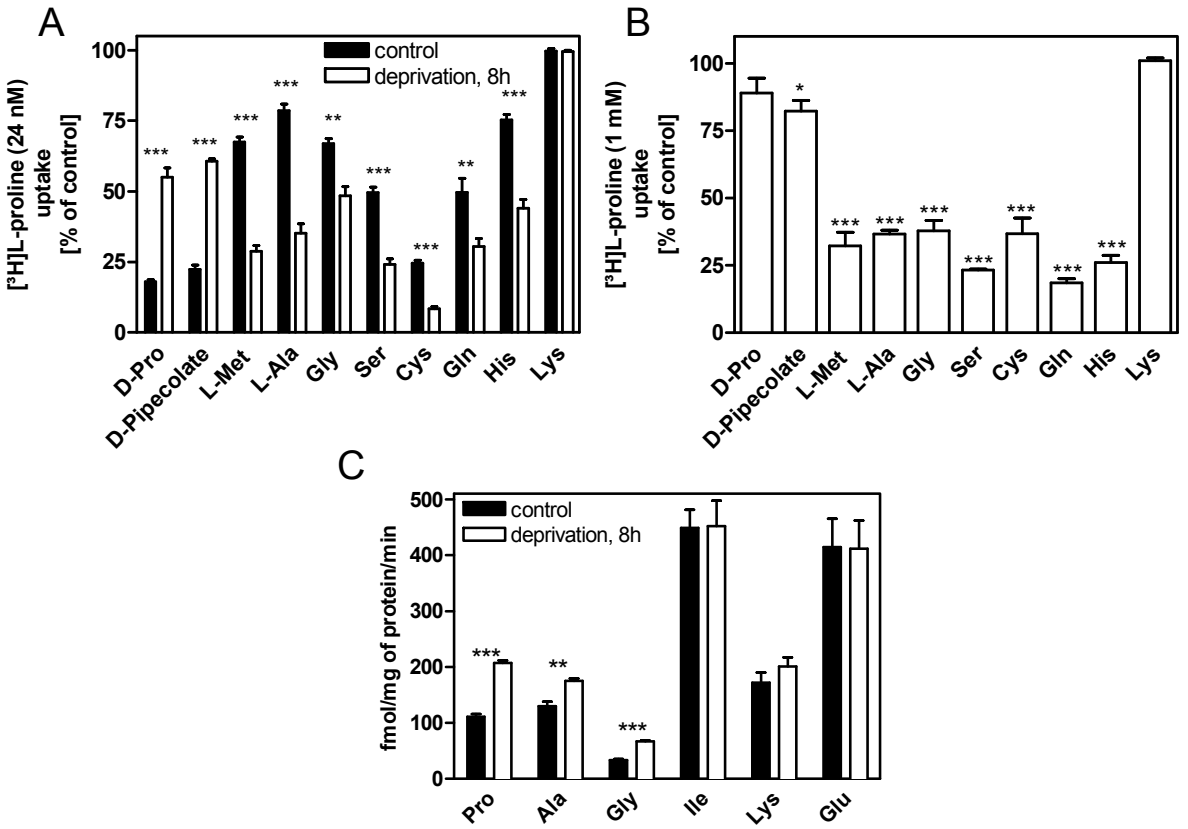

Fig. 17 - Substrate specificity of the deprivation-induced L-proline uptake. Proline uptake (24 nM, A; 1 mM, B) in the absence or the presence of different SIT1 or SNAT2 substrates or lysine provided in concentrations of 1 mM (L-Ala and L-Met) or 10 mM (all other amino acids). (C) Transport of selected 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 8h under amino acid deprivation (white bars) (n = 4). Significant differences are denoted as * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ (n = 3 to 5).

1.2.8.7 Changes of mRNA levels

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. Therefore, apparent mRNA levels of SNAT2 in comparison to those of SIT1 during amino acid deprivation were determined by RT-PCR. After a deprivation period of 4h, the mRNA level of SIT1 remained unchanged, whereas the transcript level of SNAT2 increased significantly almost 3-fold. After 8h, the transcript levels of SIT1 were increased to 1.6-fold, whereas the mRNA level of SNAT2 decreased slightly from three to two-fold over that in control cells. This decrease of mRNA levels from the 4h to the 8h incubation period could be due to an inhibitory feedback of transcription by the newly synthesized functional proteins.

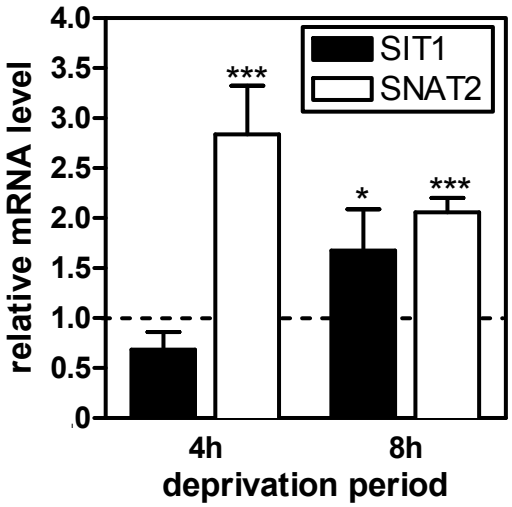

Fig. 18 - mRNA levels of SIT1 and SNAT2 genes in cells under amino acid deprivation. mRNA levels were determined by real-time RT-PCR using SYBR Green I with Roche's Light Cycler and are given as relative factor to the amount in non-deprived cells (1.0). (n = 4; data represent two independent duplet replications). *p < 0.05, ***p < 0.001 versus non-deprived cells.

1.2.8.8 Regulation of deprivation-induced influx

Since SNAT2 activity has been shown to be altered by signalling processes involving JNK, PI-3-kinases and GSK-3, proline influx after deprivation was determined 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. 19).

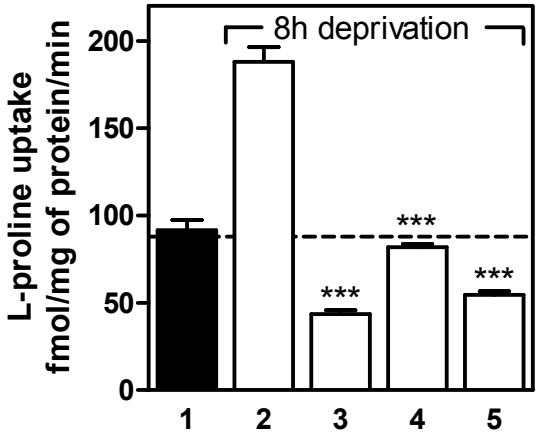

Fig. 19 - Effects of selected protein kinase inhibitors on deprivation-induced L-proline uptake. Tracer proline influx into cells grown under standard conditions or under amino acid deprivation for 8h in the absence or presence of 50 µM of JNK inhibitor II (bar 3), 50 µM of the GSK inhibitor SB-415286 (bar 4) or 100 nM of the PI-3 kinase inhibitor wortmannin (bar 5). Significant differences are denoted as *** p < 0.001 (n = 3 to 5).

1.3 Discussion

Here, for the first time a pronounced upregulation of L-proline influx into renal epithelial cells upon an amino acid deprivation is demonstrated. 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.

By RT-PCR the mRNA of SIT1, PAT1 and PAT2 could be detected expressed in OK cells. From these experiments, one must speculate that these proteins could contribute to the net proline uptake. The proline uptake was analyzed quantitatively by incubating the cells in increasing concentrations of L-proline. By a fitted Michaelis-Menten kinetic, a high affinity for the proline uptake in OK cells with a low K_m of 0.22 mM could be described. This transport rate is typical for the high-affinity SIT1 transporter. But also PAT2 shares a high affinity of 0.12 mM (Boll *et al.*, 2002) for proline while the affinity of PAT1 for L-proline is about 10-fold higher. L-proline uptake occurs in a Na^+- and Cl^--dependent manner, very poor transport of L-proline could be detected when Na^+ was replaced by Li^+.

By varying the uptake conditions and competition assays with different substrates, the proline uptake in OK cells was further investigated. Decreasing the pH from 7.4 to 6.5 and 6.0 reduced strongly the activity. Additionally, an H^+-dependent and at the same time Na^+-independent L-proline transport could not be detected. Furthermore, 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. Moreover, B^0AT2 as a candidate transporter could be excluded since it has a high affinity for methionine, isoleucine and leucine as shown by Bröer et al., whereas only a low-affinity type inhibition by these amino acids was observed (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. Besides, B^0AT1 as a Na^+-dependent transporter for proline with a low affinity could also be excluded due to the strong reduction of transport, when Na^+ was replaced by Li^+ because B^0AT1 is a Li^+ -tolerant transporter (Camargo *et al.*, 2005).

However, L-proline transport could be inhibited by all classical SIT1 substrates identified so far. In analogy to data provided by Ristic et al. for the OK SIT1 homologue (Ristic *et al.*, 2006) the ability of some more hydrophobic amino acids to reduce [^{3}H]L-proline uptake when provided in high concentrations could be detected, yet, these amino acids have a much lower affinity than proline.

As earlier described, a long-term administration of vigabatrin, a well defined PAT substrate (Abbot *et al.*, 2006) with a strong structural similarity to GABA and anticonvulsant, which is

used in treatment of resistant epilepsy, leads to an increased urinary excretion of the amino acids β-alanine, β-aminoisobutyric acid, alanine and GABA (Lahat *et al.*, 1999). Here it was investigated whether vigabatrin is an inhibitor of the proline transporter and if it is a candidate for aminoaciduria after administration. The results show that vigabatrin could inhibit L-proline transport poorly, so it is not involved in vigabatrin induced aminoaciduria and therefore, an involvement of SIT1 could also be excluded.

Because of its broad substrate specificity, it was postulated that SIT1 in vivo is not only responsible for the uptake of L-proline but also for other neutral and hydrophobic amino acids (Ristic *et al.*, 2006). Exemplarily, the transport of [^{3}H]L-alanine in the absence or presence of L- proline, a concentration saturating SIT1, was investigated. Total alanine uptake at a high and a low concentration of L-proline was independent from SIT1 activity. Therefore, in vivo SIT1 has a negligible role in reabsorption of other amino acids. Due to its high affinity for L-proline, SIT1 is already saturated at low proline concentrations in primary filtrate and so not available for transport of a bulk of neutral amino acids.

The results show that the proline transporter SIT1 is one candidate for the cause of iminoglycinuria. This disorder is characterized by a selective urinary loss of the imino acids proline, hydroxyproline and glycine due to a defect in renal tubular reabsorption (Rosenberg *et al.*, 1968): proline and hydroxyproline are high-affinity SIT1 substrates, while glycine is only a low-affinity substrate. The excretion of glycine from iminoglycinuric patients is not solely explainable by the loss of SIT1 activity. The PAT proteins would be good candidates responsible for the glycine reabsorption in proximal tubule cells. Although mRNA of PAT1 and PAT2 could be detected in OK cells, there is no functional evidence for the expression in the apical membrane, possibly the PAT proteins are localized in lysosomes as shown in brain of rats (Sagne *et al.*, 2001).

Taken together, the properties found for proline influx in OK cells with a high affinity for proline, a characteristic ion-dependence and a distinct substrate selectivity suggest that SIT1 is the predominant transporter in these cells when grown under standard conditions. Thus, OK cells are an appropriate model to study iminoglycinuria by eliminating SIT1 activity through RNAi techniques and continuative investigations of amino acid handling, especially for proline and glycine.

Next, for further characterization of proline transport, OK cells were submitted to an amino acid deprivation period of up to 8h. Now 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 as candidates 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.

This sodium-coupled neutral amino acid transporter is a member of the *SLC38* family with widespread expression, including kidney epithelium. The Na^+-dependence but also Li^+-tolerance is a hallmark of SNAT2 and was also observed in muscle, the human hepatoma cell line HepG2, neurons and 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 but also proline as substrates, whereas D-amino acids showed only very low affinity. This resembles my 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; Hyde *et al.*, 2002; Ling *et al.*, 2001) including the apical membrane of polarized cells. Since uptake in polarized cells across the apical side was assessed here, 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 2- to 3-fold 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. Jones et al. observed two phases of upregulation of transport by SNAT2: first, relocalization of existing SNAT2 transporters occured within 30 min of amino acid deprivation and continued throughout the 6h-incubation (Jones *et al.*, 2006). The second phase involved RNA and protein synthesis, which became apparent after about 3h in deprived medium but increased over the next 6h. Here, an upregulation of SNAT2 transport after 4h could not be observed, so an endosomal recruitment of SNAT2 transporters to the apical membrane could be excluded. In OK cells, the adaptive response is completely dependent on *de novo* protein synthesis. This corresponds well with the later observed involvement of RNA and protein synthesis in BeWo cells (Jones *et al.*, 2006). 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 3-fold and amino acid uptake four-fold 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 3-fold (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).

Restoration of SNAT2 activity by individual amino acids under amino acid deprivation conditions has also been described (Boerner and Saier, 1985; Kashiwagi *et al.*, 2009) and these findings are in line with these observations as proline alone partially and a mixture of Ala, Val, Ile, Phe, Cys and Ser provided under deprivation conditions completely suppresses the adaptive response in OK cells. Likewise, Jones et al. observed a reduced SNAT2 staining at the cell surface of BeWo cells after replenishing the amino acid supply, indicating the recycling of the transporters back to the perinuclear region (Jones *et al.*, 2006).

Additionally, it was shown that the effect of amino acid deprivation is specific for amino acid transport and not a general change in the physical properties of the cell layer: the transport rates of isoleucine, lysine and glutamate remained unchanged, whereas transport of proline, alanine and glycine was increased. The findings of Fong et al., who showed that the adaptive increase in amino acid transport system A does not reflect a general cell response, since the activity of other amino acid transporting systems is either uneffected or suppressed during amino acid deprivation (Fong *et al.*, 1990), support my conclusion. In NBL-1 cells, a renal bovine epithelial cell line derived from distal tubule, the high-affinity Na^+-dependent glutamate transport system X^-_{AG} was induced after an amino acid deprivation period of 24h. The V_{max} of aspartate transport was doubled, while the K_m remained unchanged (Nicholson and McGivan, 1996). Accordingly, the unchanged transport of glutamate after a clearly shorter deprivation period of 8h in OK cells can be explained.

In summary, in this part of my work 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 are described. The adaptive response involves *de novo* synthesis of new transporters and a change in kinetics of transport as well as substrate specificity that all together 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 ca-

pacity when cells are deprived of amino acids. These findings demonstrate that a rapid response to changes in renal amino acid handling is given by upregulation of the SNAT2 transport protein to increase the capacity of the renal epithelium for reabsorption and amino acid conservation under fasting and amino acid deprivation conditions.

To further improve the findings, the protein levels of the investigated transporters should be determined if antibodies recognizing the opossum transporters are available. Another important point would be the investigation of transport from the basolateral side under amino acid deprivation since mRNAlevels of SNAT2 were increased after 4h but the luminal transport remained unchanged.

2 Characteristics of transport of selenoamino acids in renal and intestinal cells

Since amino acids such as methionine and cysteine are found in nature also with selenium replacing the sulphur in the molecule, it was one of the goals of this project to characterize the transport of selenium-containing amino acids and derivatives for uptake into the intestine or for reabsorption in the renal tubular system.

2.1 Chemical properties of selenium

Selenium is a chemical element with the atomic number 34 and is represented by the chemical symbol Se. It is a non-metal and chemically related to sulphur. It is toxic in large amounts (> 400 µg per day), but trace amounts (55 µg per day for adults) are essential for humans. Selenium forms the active center of the enzymes glutathione peroxidase and thioredoxin reductase (Behne and Kyriakopoulos, 2001), which are primarily responsible for reducing peroxide free radicals that include lipid peroxides in cell membranes. Thus, selenium contributes to antioxidative defense in cells. Furthermore, selenium is a component of deiodinase enzymes, which convert thyroid hormones into active or inactive forms (Arthur, 1991).

Selenium rarely occurs in its elemental state in nature but has several inorganic forms, including selenide, selenate and selenite. Selenium is like sulphur and as such plants and bacteria are able to synthesize organic selenium compounds from nearly all naturally occuring organic selenium sources such as the seleno-derivatives of methionine and cysteine (Schrauzer, 2004). Selenomethionine is synthesized in dependence on the availability of sulphur or selenium without any structural or functional impairments or any genetical control (Schrauzer, 2004).

Food sources of selenium are meats, fish, shellfish, grain, garlic, onions and eggs. The importance of an adequate supply of selenium was investigated inter alia in many epidemiological studies. They suggest that a low selenium supply by the diet is associated with an increased prevalence of various degenerative diseases and an increased risk of cancer (Combs and Gray, 1998; el-Bayoumy *et al.*, 1995; Schrauzer *et al.*, 1977). Consequently, selenium supplementation is widely used. The main sources of food selenium are organic selenium compounds such as dimethyl selenide, selenomethionine (Se-Met), selenocysteine, methylselenocysteine (MSC) and selenocystine (Se-Cys) for which the structures are shown in Fig. 20. These compounds have a high bioavailability (Daniels, 1996; Swanson *et al.*, 1991) and a rather low toxicity. Other selenium compounds that are also found in supplements are inorganic salts like selenate. Several organoselenium compounds, including selenobetaine (Se-Bet) and methylated selenoamino acids like MSC, were found to possess

anticarcinogenic activity in cell cultures and tumor models and these compounds show generally lower toxicity than anorganic selenium (Ip and Ganther, 1992).

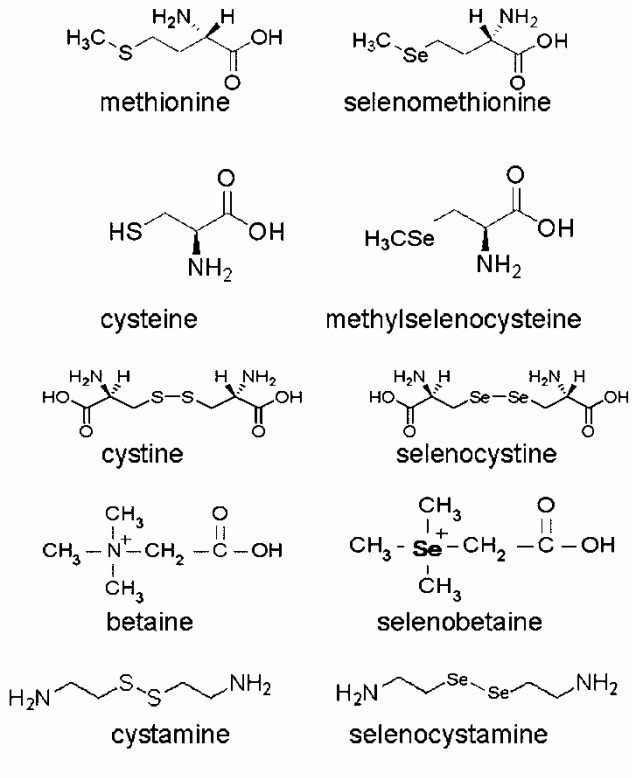

Fig. 20 - Selenium compounds and sulphur analogs.

2.2 Intestinal absorption of selenium compounds

The absorption of selenium depends on the structure of the compound. Selenium is efficiently absorbed in the upper part of the small intestine, organic selenium compounds from vegetable and animal food, e.g. selenomethionine and selenocysteine, show a intestinal bioavailability of 90%, whereas simply 60% of anorganic selenium compounds, like Na^+ - selenide, contained in dietary supplements, is absorbed (Ekmekcioglu, 2000). Intestinal availability is synergistic with vitamin E and A, whereas heavy metals seem to inhibit the absorption of selenium (Ekmekcioglu, 2000).

The intestinal absorption of anorganic and organic selenium compounds occurs via different routes and by different mechanisms. Selenate shares a sodium-dependent transport system with sulphates, whereas selenite is mainly absorbed by passive diffusion (Vendeland *et al.*, 1992). Uptake of selenoamino acids and their sulphur analogs has been studied in brush border membrane vesicles (BBMV), but the results are partially contradictory. Wolffram et al. showed in porcine intestinal BBMV that Se-Met and its sulphur analog are transported in a Na^+-dependent fashion with similar maximal transport velocities and Michaelis constants

(Wolffram *et al.*, 1989). Furthermore, Se-Cys was found to share a common transport system with cystine and basic amino acids (Wolffram *et al.*, 1989). By contrast, McConnell et al. reported a passive absorption of Se-Cys in everted intestinal sacs (McConnell and Cho, 1965) and Vendeland et al. also suggested a passive transport mechanism, since the initial uptake of selenite, Se-Met and selenate in BBMV from rat small intestine was linear over a concentration range of 10 – 1000 µM (Vendeland *et al.*, 1992). Leblondel et al. however reported that transport of Se-Met is inhibited by its sulphur analog in Caco-2 cells, suggesting a common transport route, but an inhibition of transport of cysteine by Se-Cys was not observed (Leblondel *et al.*, 2001).

2.3 Aim of this work

Despite numerous amino acid transporter genes have been cloned and characterized in the last decade, transport of selenoamino acids by distinct epithelial amino acid transporters has not yet been studied in detail. Moreover, renal reabsorption of selenium compounds has not been investigated so far, although kidney, followed by liver, testes and spleen shows generally highest tissue selenium concentrations (Behne *et al.*, 1988; Oster *et al.*, 1988).

It was the intention of this project part to characterize the uptake of selenoamino acids Se-Met, MSC, Se-Cys, Se-Bet, selenocystamine and their sulphur analogs, employing the OK and Caco-2 epithelial cell lines. OK cells are known to express endogenously the amino acid transporters SIT1 (xT3s1 in rodents), $b^{0,+}$rBAT, and B^0AT1 (Ristic *et al.*, 2006) as described in chapters 1.1.3.1 and 1.1.4.3. The Caco-2 cell line is an immortalized line of heterogeneous human epithelial colorectal adenocarcinoma cells developed by the Sloan-Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh (Fogh *et al.*, 1977). They exhibit after differentiation typical characteristics of enterocytes (Chantret *et al.*, 1988; Delie and Rubas, 1997). Caco-2 cells do not express SIT1, but have PAT1 (Metzner *et al.*, 2004) and also $b^{0,+}$rBAT as well as B^0AT1 (Ferruzza *et al.*, 1995; Kekuda *et al.*, 1997) in their apical membrane. Furthermore, the fate of the selenium compounds in the cell should be investigated and which amino acid transporters mediate their cellular uptake should be analyzed. For this, the amino acid transporters B^0AT1, $b^{0,+}$rBAT, SIT1, and PAT1 were heterologously expressed in the *Xenopus laevis* oocyte system with recording of transport currents as a function of substrate concentrations.

2.4 Results

2.4.1 Transport measurements in oocytes

2.4.1.1 Interaction of selenoamino acids with B^0AT1

Fig. 21A shows the I-V relationship for leucine uptake into oocytes expressing B^0AT1. Leucine is used here as a reference substrate. Increasing concentrations of methionine also generated currents similar to those of leucine. Substrate affinities were calculated at -60 mV membrane potential and revealed an apparent K_m value of 0.67 ± 0.10 mM for methionine and 0.61 ± 0.13 mM for Se-Met (Fig. 21B and Table 2). The calculated maximal transport current (I_{max}) for Se-Met was slightly (13 ± 5 %) lower than that of leucine, while the maximal currents generated by methionine and leucine were identical. Transport parameters for MSC and cysteine transport revealed mean K_m values of 0.80 ± 0.06 mM and 1.40 ± 0.33 mM, respectively, and the I_{max} values of both compounds were identical with that of leucine (Fig. 21C). The K_m values of the selenoamino acids and their corresponding sulphur analogs did not differ significantly. Se-Bet and Se-cystamine were not transported by B^0AT1 (Fig. 21D and E). Se-Cys was not tested as cystine is not substrate for B^0AT1.

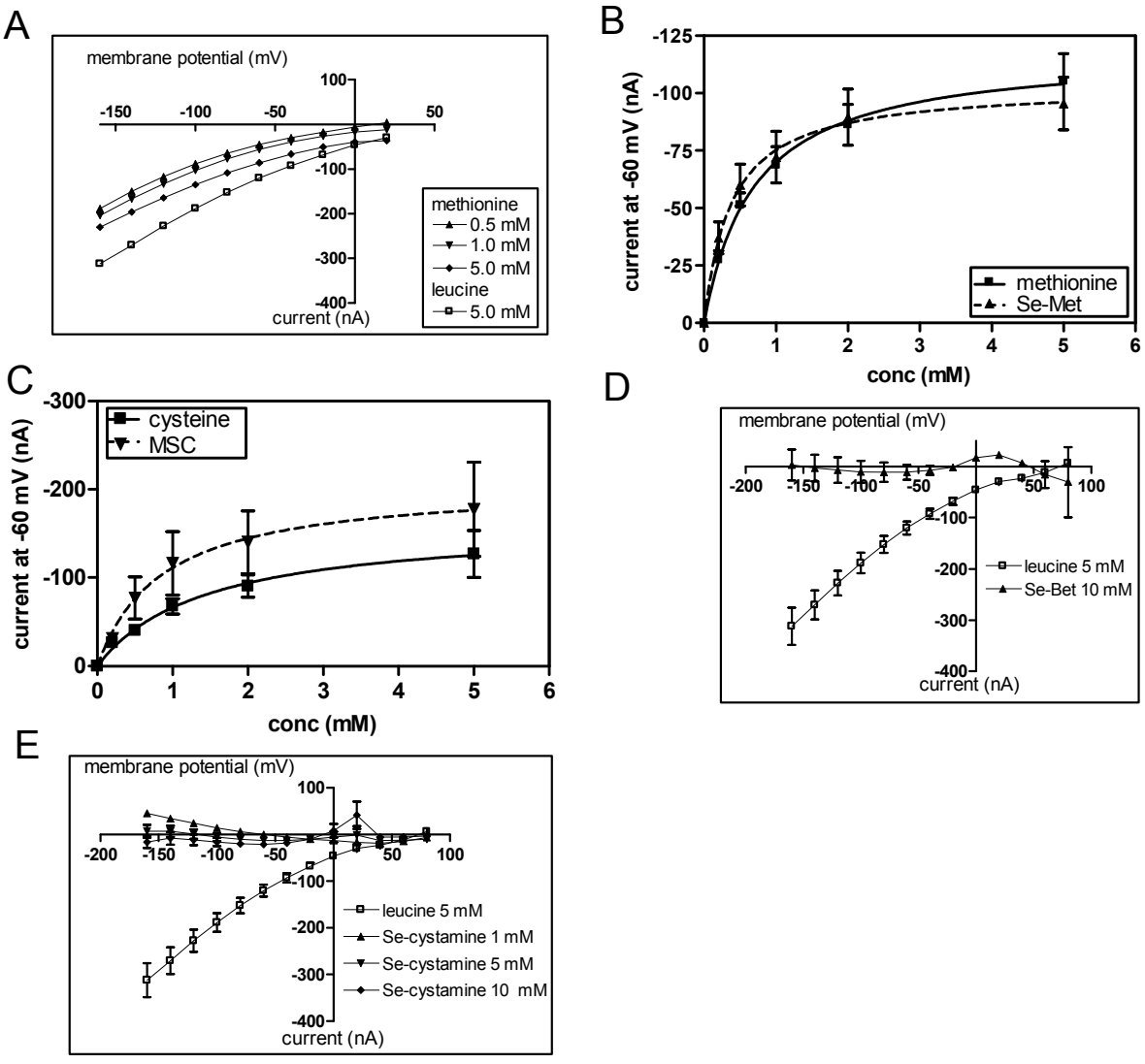

Fig. 21 - Interaction of selenoamino acids with B^0AT1 expressed in Xenopus oocytes. Transport currents were measured at -60 mV and at pH 7.5 (A) Steady-state I/V relationships measured with leucine (5 mM) or increasing concentrations (0-5 mM) of methionine. Means of 5 oocytes.

(B) and (C) Transport currents as a function of methionine or Se-Met (0-5 mM) and cysteine or MSC (0-5 mM) and the Michaelis-Menten kinetics fitted to the data points. Means ± S.E.M. of 9 and 7 oocytes, respectively. (D) and (E) Steady-state I/V relationships measured with leucine (5 mM) or Se-Bet (10 mM) and Se-cystamine (1-10 mM), respectively. Means ± S.E.M. of 2 and 3 oocytes, respectively.

2.4.1.2 Interaction of selenoamino acids with $b^{0,+}$rBAT

As described in the methods section, the oocytes were voltage-clamped at -20 mV, but the K_m values were calculated at a membrane potential of +80 mV, since the outward current generated in exchange for the inward transport of uncharged substrates was found highest at this potential. The measured current shown here does not result from the inward transport of cystine, but from the countertransport of positively charged amino acids endogenously present in the oocyte. Comparison of the I/V relationships shown in Fig. 21A (B^0AT1) and Fig. 22A ($b^{0,+}$rBAT) clearly shows the differences between the symporter B^0AT1 and the exchanger $b^{0,+}$rBAT. For Se-Met, a K_m value of 0.12 ± 0.02 mM was calculated and a nearly equal K_m value of 0.13 ± 0.02 mM for methionine (Fig. 22B; Table 2). The calculated I_{max} was slightly (14%) lower for Se-Met. MSC and cysteine revealed K_m values of 0.20 ± 0.03 mM and 0.48 ± 0.06 mM, respectively, but the I_{max} values were not significantly different (Fig. 22C). L-cystine was transported by the $b^{0,+}$rBAT system with an affinity of 0.11 ± 0.03 mM, its selenium analog showed a significantly ($p < 0.05$) higher K_m value of 0.22 ± 0.05 mM (Fig. 22D). However, the measured current shown here does not result from the inward transport of cystine, but from the countertransport of positively charged amino acids endogenously present in the oocyte. The I_{max} values for cystine and Se-Cys were only 54 ± 2% and 65 ± 4% of the leucine-induced maximal currents measured in the same oocytes.

Transport at +80 mV membrane potential confirms, that the investigated substrates can bind to and can be translocated by the transporter, but if under physiological conditions seleno-amino acids will be taken up by the $b^{0,+}$rBAT system into the cell, it will depend on the actual membrane potential and the relative concentrations of the involved amino acids. Nevertheless, under the present experimental conditions, some small inward transport could be seen also at -20 mV and at this potential the K_m values were estimated only 2 to 3 times higher than at +80 mV.

Se-Bet (Fig. 22E) did not show any transport activity, but showed some unspecific effects (irregular I-V relations at high positive membrane potentials), which were present only to a lesser degree in non-injected oocytes. This effect could be due to the positive charge as the I-V relations of arginine show a similar characteristic at high positive membrane potentials. However, this effect was not dependent on the concentration and so could not be seen as transport.

Selenocystamine (Fig. 22F) did not induce any current at positive membrane potentials and was therefore not transported.

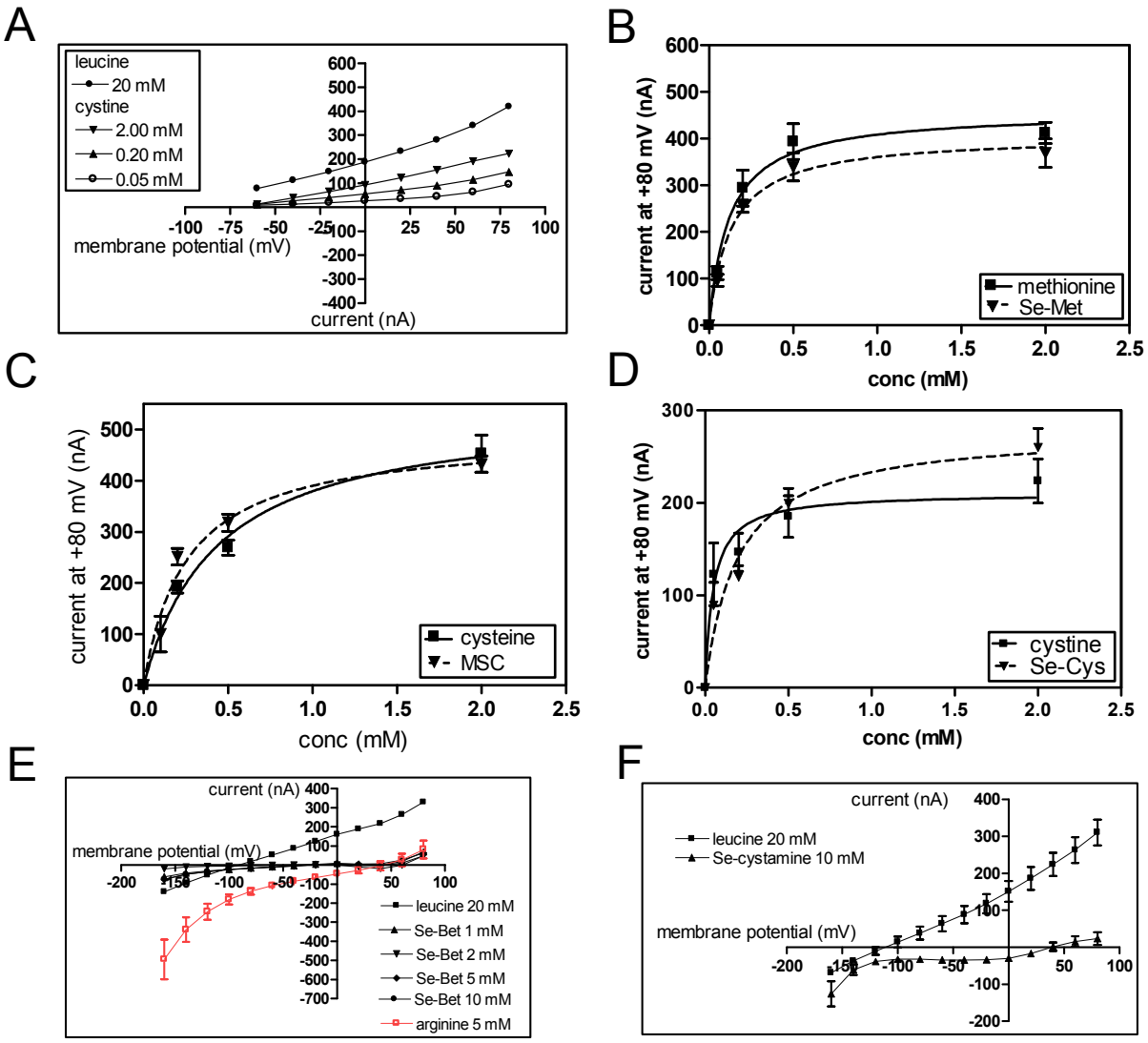

Fig. 22 – Interaction of selenoamino acids with $b^{0,+}$rBAT expressed in Xenopus oocytes. Transport currents were measured at +80 mV and at pH 7.5 (A) Steady-state I/V relationships measured with leucine (20 mM) or increasing concentrations (0-2 mM) of cystine. Means of 7 oocytes. (B)-(D) Transport currents as a function of methionine or Se-Met (0-2 mM), cysteine or MSC (0-5 mM), cystine or Se-Cys (0-2 mM) and the Michaelis-Menten kinetics fitted to the data points. Means ± S.E.M. of 7 oocytes.
(E) and (F) Steady-state I/V relationships measured with leucine (20 mM), arginine (5 mM), Se-Bet (1-10 mM) and Se-cystamine (10 mM), respectively. Means of 3 oocytes for Se-Bet, Means ± S.E.M. of 3 oocytes for Se-cystamine.

2.4.1.3 Excursus: stimulation of heteromeric exchange by injection of amino acids

The heteromeric amino acid transporters are obligate exchangers, transport only happens in the presence of extra- and intracellular amino acids. Obviously, there are high enough concentrations of both neutral and positively charged amino acids in the oocyte to allow the transport, when uncharged or positively charged substrates are offered on the extracellular side. However, if the exchange can be augmented by increasing the intracellular amino acid concentrations via direct injection was not yet investigated. Therefore, oocytes were injected

with leucine or arginine with amounts high enough to produce cytosolic concentrations of about 20 mM.

After the injection of leucine, a 4.8-fold increase of arginine-induced inward current at -60 mV was detected, whereas the leucine-induced outward currents were reduced by 35% at a membrane potential of +60 mV (Fig. 23A). On the other hand, the injection of arginine led to a decrease of arginine-induced currents by 47% at -60 mV and a stimulation of leucine-induced outward currents by 48% at +60 mV (Fig. 23B).

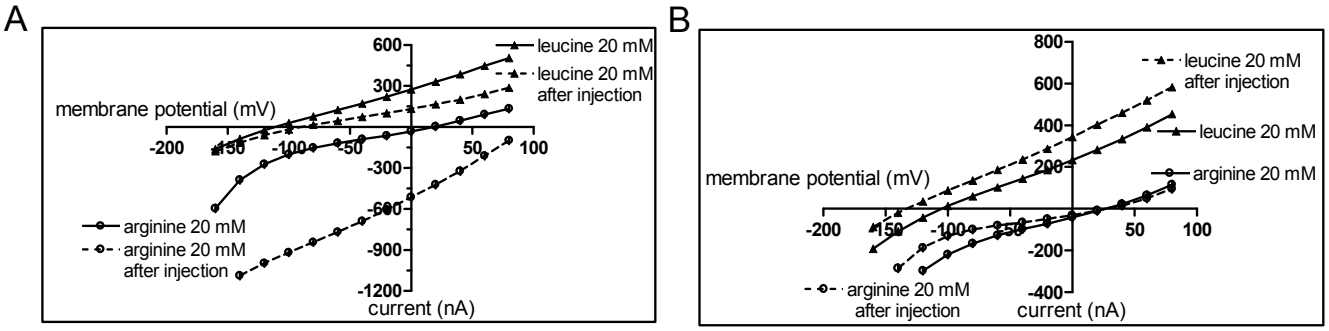

Fig. 23 - Stimulation of heteromeric exchange by injection of amino acids. (A) Steady-state I/V relationships of preinjected (20 mM leucine) or non-injected oocytes at a holding potential of -20 mV at pH 7.5 were measured in the presence of leucine (20 mM) or arginine (20 mM). Data are presented as means from one experiment (n = 3).
(B) Steady-state I/V relationships of preinjected (20 mM arginine) or non-injected oocytes at a holding potential of -20 mV at pH 7.5 were measured in the presence of leucine (20 mM) or arginine (20 mM). Data are presented from one experiment.

2.4.1.4 Interaction of selenoamino acids with SIT1

In this chapter, it was analyzed whether selenoamino acids and their sulphur analogs are substrates of SIT1. Fig. 24A shows the I-V relationship for proline uptake into oocytes expressing SIT1. Proline is used here as a reference substrate. Increasing concentrations of methionine also generated currents similar to those of proline except for the concentrations 0.5 and 1 mM, which are far below the K_m for this substrate. Substrate-evoked inward currents could be recorded for all selenoamino acids except for selenocystamine (Fig. 24F). For Se-Met, a K_m value of 3.69 ± 0.28 mM, and for methionine a K_m value of 6.93 ± 0.66 mM was obtained (Fig. 24B). MSC and cysteine revealed K_m values of 0.47 ± 0.02 mM and 2.64 ± 0.14 mM (Fig. 24C), respectively. Se-Bet possessed only a low affinity for SIT1 (K_m= 9.37 ± 0.75 mM) as compared to betaine with a K_m of 0.17 ± 0.02 mM (Fig. 24D and E).

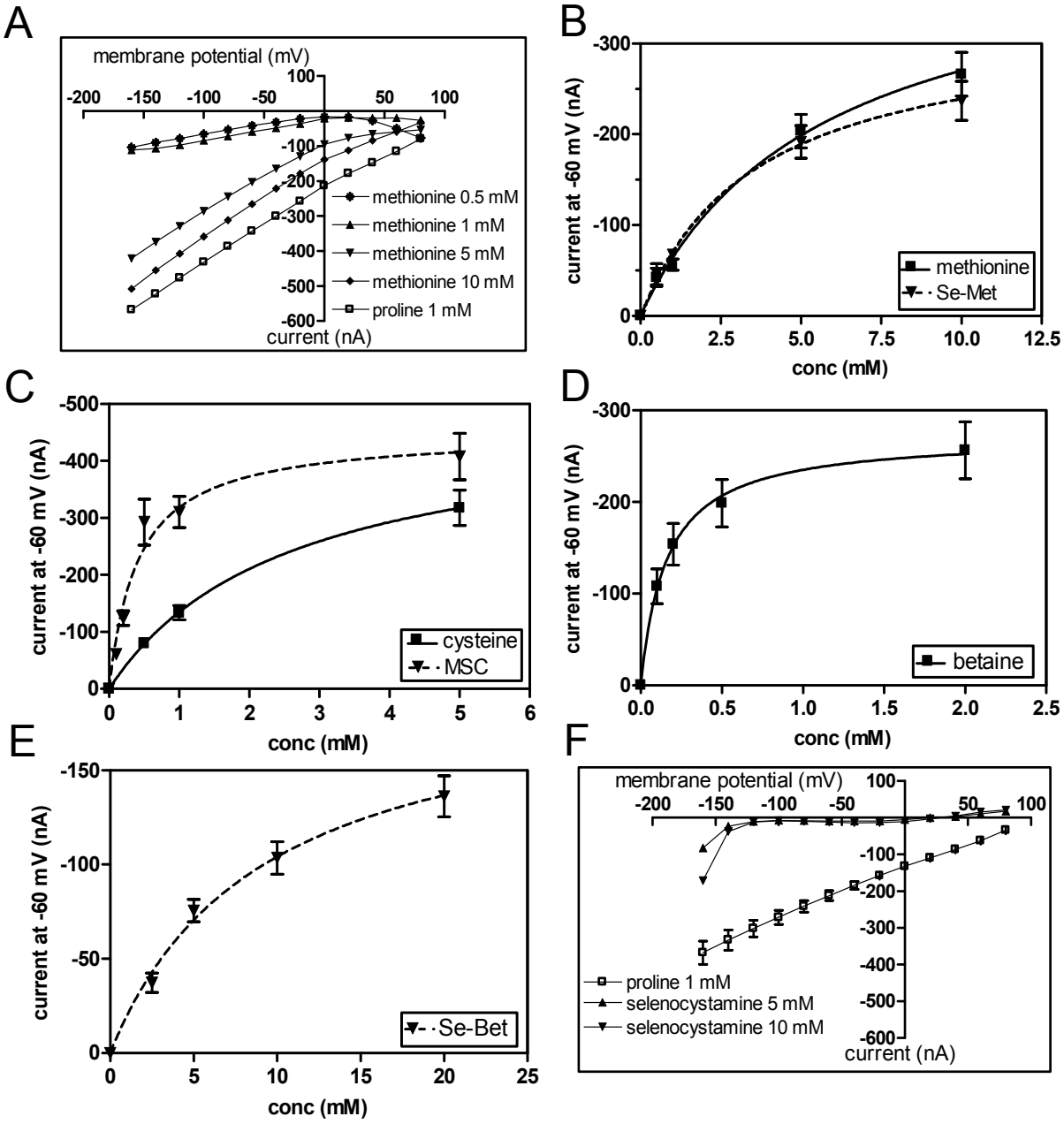

Fig. 24 - Interaction of selenoamino acids with SIT1 expressed in Xenopus oocytes. Transport currents were measured at -60 mV and at pH 7.5 (A) Steady-state I/V relationships measured with proline (1 mM) or increasing concentrations (0-10 mM) of methionine. Means of 5 oocytes.
(B)-(E) Transport currents as a function of methionine or Se-Met (0-10 mM) (n = 9), cysteine or MSC (0-5 mM) (n = 9), betaine (0-2 mM; n = 4) or Se-Bet (0-20 mM; n = 8) and the Michaelis-Menten kinetics fitted to the data points. Means ± S.E.M
(F) Steady-state I/V relationships measured with proline (1 mM) or Se-cystamine (5 or 10 mM, n = 5).

2.4.1.5 Interaction of selenoamino acids with PAT1

PAT1 is a H^+-coupled transporter for glycine and other small neutral amino acids. To provide an adequate H^+ -gradient, this experiment was done at an outside pH of 6.5. Measuring the substrate-evoked currents in oocytes expressing PAT1, Se-Bet induced currents, but transport showed only a very low affinity > 20 mM (Fig. 25A). This could also be reproduced by

using competition assays on the intestinal cell line Caco-2 as a model for PAT1 (Metzner *et al.*, 2004): it could be demonstrated that Se-Bet is a worse substrate than betaine, but both showed a rather low inhibition of L-proline uptake (inhibition of 24%, K_m of > 20 mM for Se-Bet at a concentration of 20 mM and an K_m of 5.4 ± 0.1 mM for betaine, respectively, B).
For all other amino acids and selenium analogs, no substrate-evoked inward currents could be recorded (data not shown).

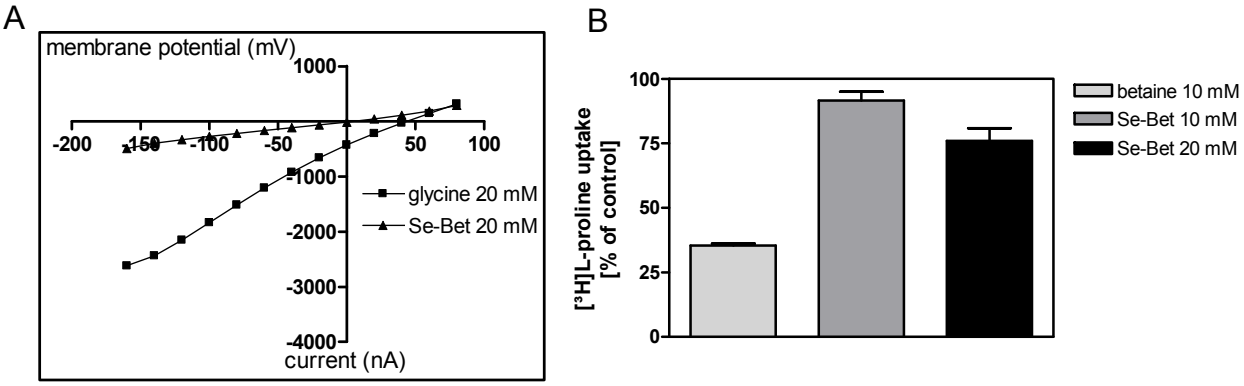

Fig. 25 - Interaction of selenoamino acids with PAT1. (A) Steady-state I/V relationships measured with glycine (20 mM) or Se-Bet (20 mM) of methionine. Means of 4 oocytes. (B) Interaction of betaine or selenobetaine with hPAT1 in Caco-2 cells. Uptake of [³H]L-proline (50 µM) was measured at pH 7.4 for 10 min in the presence or absence of betaine (10 mM) or selenobetaine (10, 20 mM). The uptake rate measured in the absence of inhibitors (55.6 ± 9.13 pmol/mg of protein/min) was taken as 100%. Data are presented as means ± S.E.M. ($n_{betaine}$ = 3; $n_{Se\text{-}Bet\ 10\ mM}$ = 6; $n_{Se\text{-}Bet\ 20\ mM}$ = 4)

2.4.2 Transport measurements in cells

2.4.2.1 Determination of selenoamino acids in cells by LC-MS/MS

In this series of experiments, OK and Caco-2 cells were exposed to the different selenoamino acids (2 mM) and the intracellular contents were quantified by LC-MS/MS.
Fig. 26 shows the LC-MS/MS spectra of the standard solutions of the organic selenium compounds. After incubation, the following intracellular concentrations were found (nmol/mg of protein, Fig. 27): Se-Met in OK cells 77.3 ± 10.5, in Caco-2 cells 7.0 ± 2.4; MSC in OK cells 90.8 ± 9.0, in Caco-2 cells 11.3 ± 2.0; Se-Cys in OK cells 74.0 ± 17.9, in Caco-2 cells 46.8 ± 11.7. For Se-Bet, nearly equal concentrations in Caco-2 (22.2 ± 5.1) and OK cells (18.0 ± 5.0) could be detected. Taken together, the intracellular contents of Se-Met and MSC in OK cells were significantly higher than those in Caco-2 cells, whereas in case of Se-Cys and Se-Bet no differences between the two cell lines were obtained.

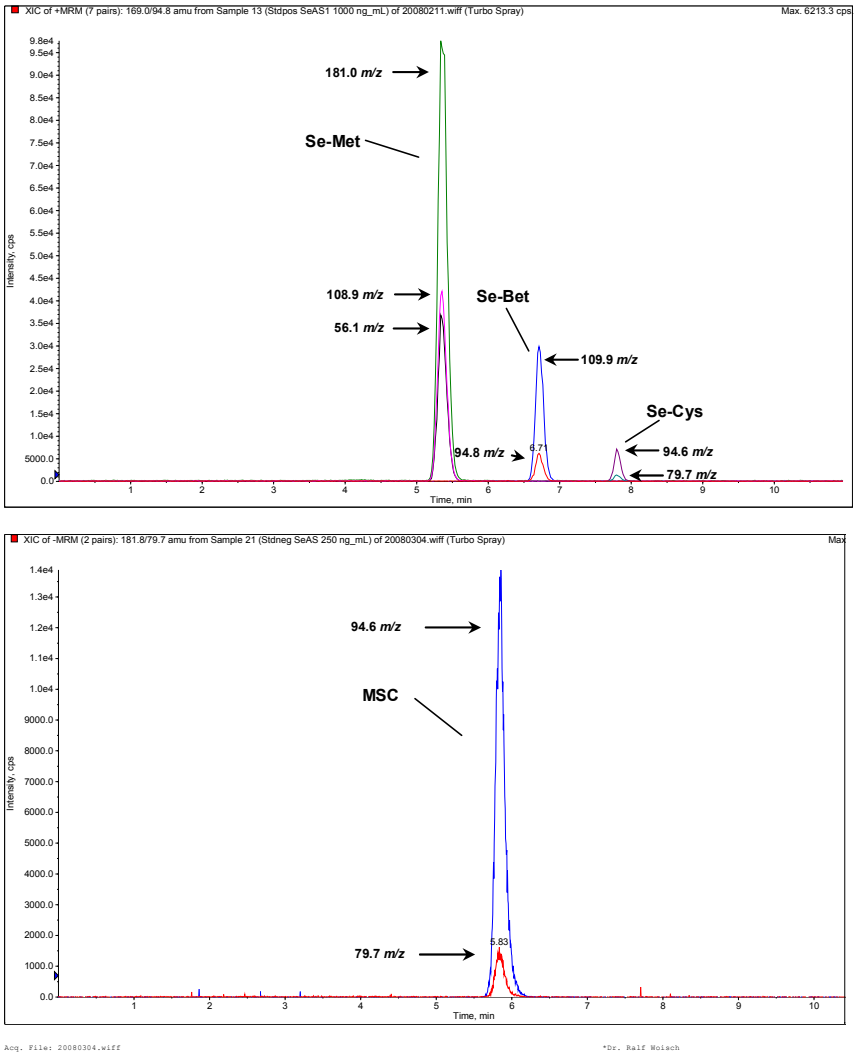

Fig. 26 - Spectra of the standard solutions of organic selenium compounds measured by using LC-MS/MS.

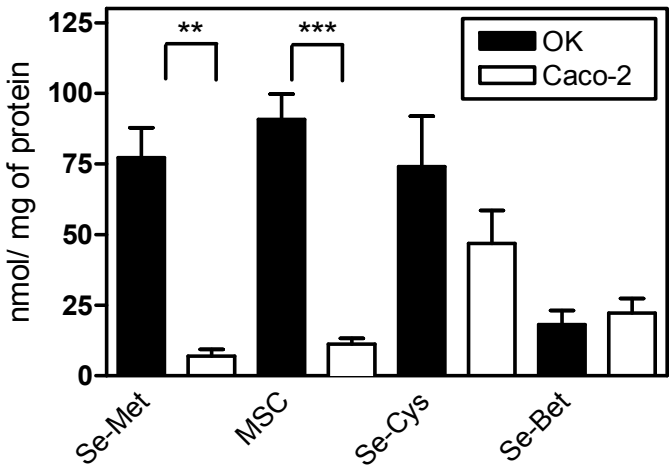

Fig. 27 - Concentrations of selenoamino acids in OK or Caco-2 cells measured by LC -MS/MS. Cells were incubated with organic selenium compounds (2 mM) for 10 min (n = 3-4). Data are presented as means ± S.E.M. **p < 0.01, ***p < 0.001.

2.4.2.2 Intracellular amino acid levels after exposure of cells to selenoamino acids

To assess whether the uptake of selenoamino acids has any effect on the cytosolic concentration of other amino acids and their derivatives, the intracellular levels of some 20 amino acids and derivatives were measured in the absence and the presence of Se-Met, MSC and Se-Bet (incubation time: 10 min) in OK cells (Fig. 28). After incubation of OK cells with Se-Met, but not after Se-Bet, the intracellular concentrations of taurine and glutamic acid decreased from 17.1 ± 0.9 to 10.8 ± 1.2 nmol/mg of protein and 38.1 ± 2.2 to 28.4 ± 2.5 nmol/mg of protein, respectively. In case of MSC incubation, taurine declined from 17.1 ± 0.9 to 11.1 ± 1.6 and glutamic acid from 38.1 ± 2.2 to 21.6 ± 5.0 nmol/mg of protein. Concentrations of all other amino acids in the cells did not change significantly.

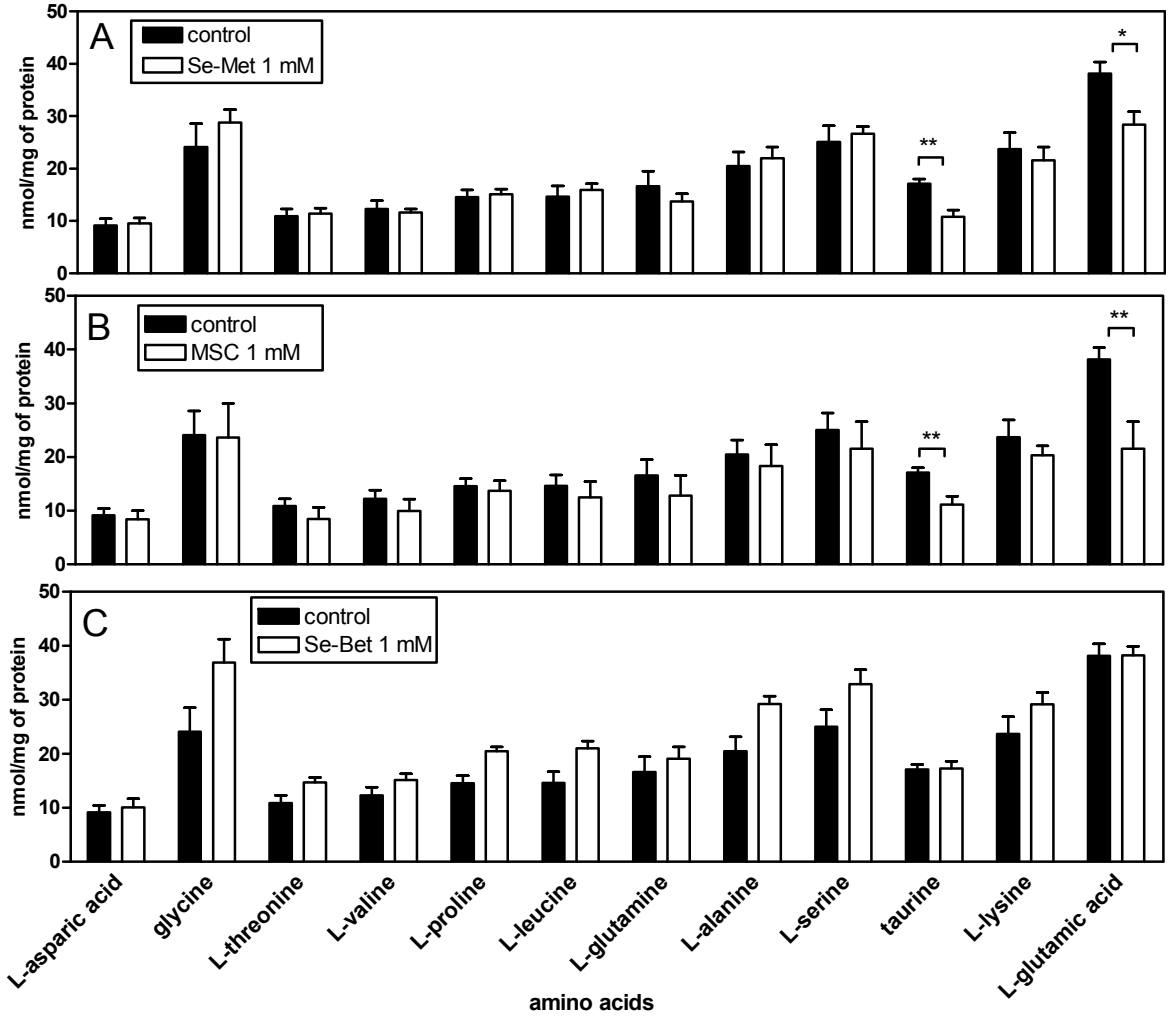

Fig. 28 - Intracellular amino acid concentrations in OK cells (n = 5) after treatment with selenoamino acids (1 mM) for 10 min. Data are means ± S.E.M.

2.4.2.3 Uptake of selenium from the test compounds into OK and Caco-2 cells

OK and Caco-2 cells were exposed to the different selenium-compounds and the cellular selenium content was determined by AAS. The apparent affinity constants for transport - based on selenium accumulation - were determined by exposure of cells to increasing concentrations of the selenium compounds as described in the Methods section.

As shown in Fig. 29 and Fig. 30, intracellular selenium contents followed Michaelis-Menten-like kinetics and allowed corresponding kinetic constants to be derived. The transport rate in Caco-2 cells was however always considerably lower than in OK cells, where Se-Met yielded a transport constant of 0.81 ± 0.47 mM (Fig. 29A), MSC of 1.43 ± 0.95 mM (Fig. 29B), Se-Cys of 1.45 ± 0.44 mM (Fig. 29C) and Se-Bet as high as 6.72 ± 7.87 mM (Fig. 29D). In Caco-2 cells, Se-Met and Se-Cys revealed high-affinity type uptake with apparent K_m values of 0.22 ± 0.07 mM and 0.32 ± 0.15 mM, respectively (Fig. 30A and C). MSC was a rather poor substrate with a K_m of 1.67 ± 1.66 mM (Fig. 30B), whereas selenium content after exposure of Caco-2 cells to Se-Bet did not obey the characteristics of Michaelis-Menten kinetics, but showed reduced levels at the highest concentration of 10 mM (Fig. 30D).

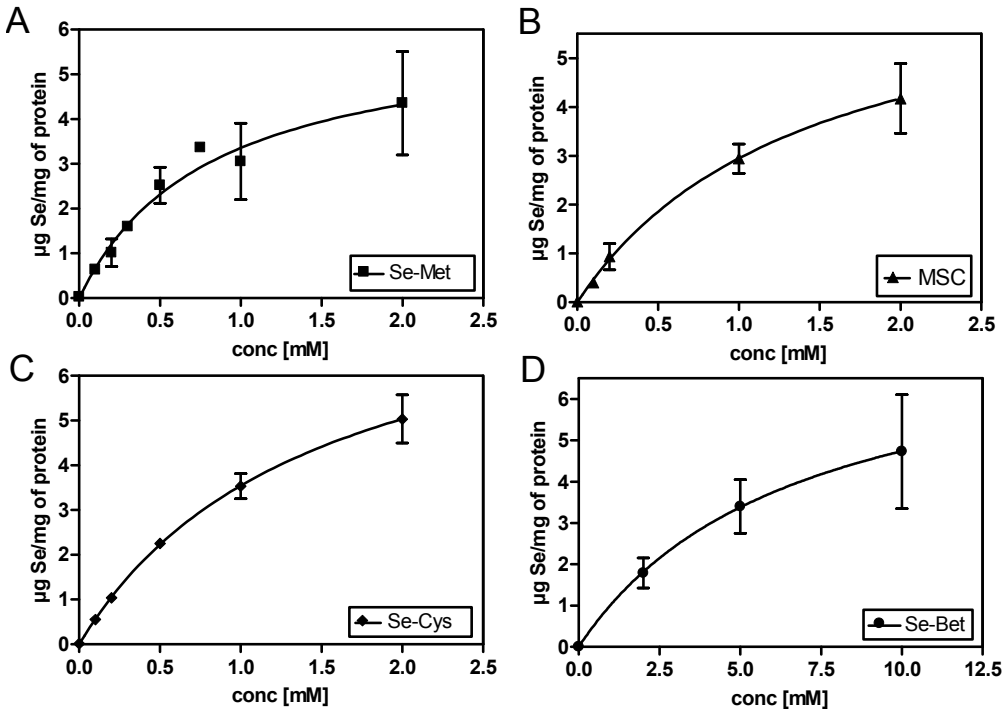

Fig. 29 - Intracellular selenium concentrations in OK cells (n = 5) after treatment with increasing concentrations (0-2 mM) of Se-Met, MSC, Se-Cys or Se-Bet (2-10 mM) for 10 min. Data are means ± S.E.M.

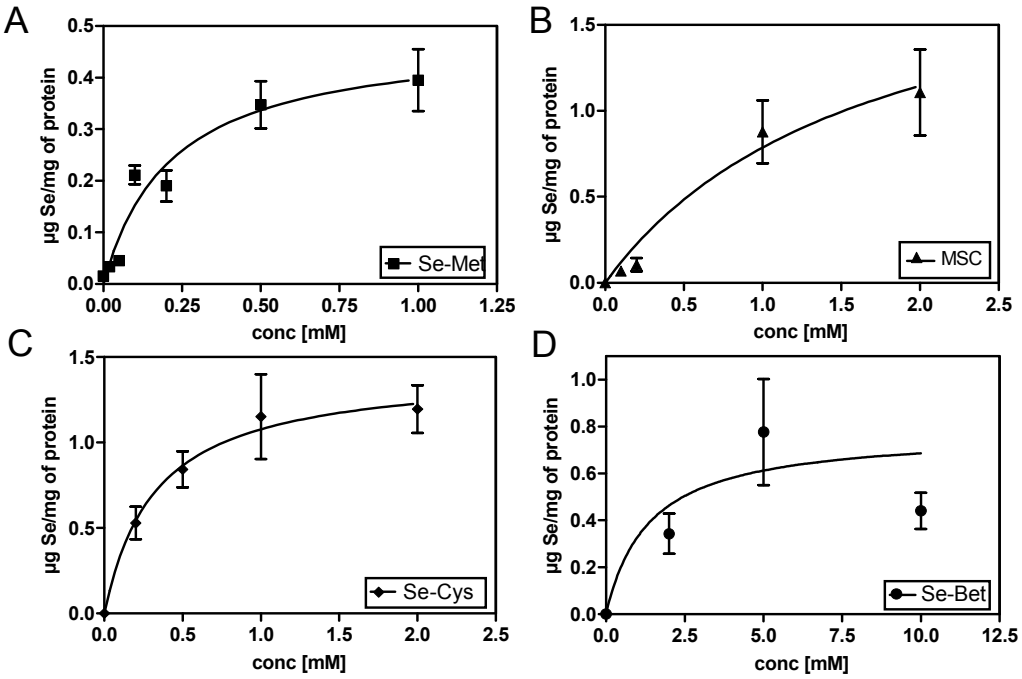

Fig. 30 - Intracellular selenium concentrations in Caco-2 cells (n = 5) after treatment with increasing concentrations of Se-Met (0-1 mM), MSC (0-2 mM), Se-Cys (0-2 mM) or Se-Bet (2-10 mM) for 10 min. Data are means ± S.E.M.

Table 2 summarizes all the results on the kinetics of interactions of the sulphur or selenium containing amino acids with the expressed transporters and those of the flux studies in the cell systems:

	Oocytes				**Cell lines**	
	B^0AT1	$b^{0,+}rBAT$	SIT1	PAT1	OK	Caco-2
Betaine	n.t.	n.t.	0.17 ± 0.02 ** 112 ± 6 %	5.4 (Boll *et al.*, 2002) n.d.	n.d.	n.d.
Selenobetaine	n.t.	Note 1	9.37 ± 0.75 90 ± 7 %	> 20 n.d.	6.72 ± 7.87 7.92 ± 4.64	Note 2
Methionine	0.67 ± 0.10 96 ± 5 %	0.13 ± 0.02 102 ± 6 %	6.93 ± 0.66 ** 125 ± 16 %	n.t.	n.d.	n.d.
Selenomethionine	0.61 ± 0.13 87 ± 5 % $^{+}$	0.12 ± 0.02 88 ± 5 % $^{+}$	3.69 ± 0.28 76 ± 6 % $^{+}$	n.t.	0.81 ± 0.47 6.08 ± 1.58	0.22 ± 0.07 0.4 ± 0.32
Cysteine	1.4 ± 0.33 * 120 ± 7 % $^{+}$	0.48± 0.06 * 107 ± 14 %	2.64 ± 0.14 ** 128 ± 7 % $^{+}$	n.t.	n.d.	n.d.
Methyl-selenocysteine	0.8 ± 0.06 114 ± 7 %	0.20 ± 0.03 93 ± 9 %	0.47 ± 0.02 114 ± 7 %	n.t.	1.43 ± 0.95 7.15 ± 2.36	1.67 ± 1.66 2.10 ± 1.11
Cystine	n.t.	0.11 ± 0.03 * 54 ± 2 % $^{++}$	n.d.	n.d.	n.d.	n.d.

Selenocystine	n.t.	0.22 ± 0.05* 65 ± 4 % ++	n.d.	n.d.	1.45 ± 0.44 8.68 ± 1.38	0.32 ± 0.15 1.43 ± 0.19
Selenocystamine	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Table 2 - Kinetic parameters on the interactions of organic selenium compounds with renal and intestinal amino acid transporters. The values shown are K_m (first line, in mM) and I_{max} (second line). The maximal transport currents in oocytes are expressed as per cent of the current generated by the saturating concentration of a known reference substrate for the given transporter (1 mM proline for SIT1, 20 mM leucine for $b^{0,+}$rBAT, 5 mM leucine for B^0AT1 and 20 mM glycine for PAT1). The transport was measured at -60 mV membrane potential for SIT1, B^0AT1 and PAT1 and at +80 mV for $b^{0,+}$rBAT. The maximal transport rate in cells is expressed as µg selenium/mg of protein * 10 minutes. n.t.: no measurable transport by the given transporter; n.d.: not determined; Note 1: no transport, but unspecific membrane effects; Note 2: cellular uptake, but no Michaelis-Menten kinetics. * and ** denote significantly different K_m values between the selenium and sulphur compounds and + and ++ denote significant differences between the compound and the reference substance (< 5% and < 1%, respectively).

2.5 Discussion

By determining substrate affinities and transport currents in oocytes expressing distinct amino acid transporting systems and by measuring selenoamino acid uptake and selenium concentrations in OK and Caco-2 cell lines, it was found that selenoamino acids share with their sulphur containing analogs the same transporters with only minor differences in substrate affinities. By comparing K_m values obtained in oocytes with those obtained in cell lines, the relative contribution of individual transport systems to overall amino acid uptake by the cells could be estimated.

Transport characteristics of the tested substrates

Both, Caco-2 and OK cells were shown to transport all seleno-compounds and accumulated selenium in the cell interior. Significantly higher intracellular contents of selenium in OK cells could be detected than in Caco-2 cells when cells were exposed to identical concentrations of Se-Met or MSC. This may indicate a higher number of transporter proteins in the apical membranes of the renal cells as compared to the intestinal cells. This high capacity for renal uptake of selenoamino acids may also explain the high concentrations of selenium that is normally found in kidney (Behne *et al.*, 1988; Oster *et al.*, 1988).

Methionine and selenomethionine

According to the data obtained in oocytes, methionine and Se-Met were transported by SIT1, $b^{0,+}$rBAT and B^0AT1, but not by PAT1. The highest affinity for Se-Met showed $b^{0,+}$rBAT, the lowest SIT1. Interestingly, SIT1 displayed a higher affinity for Se-Met than for methionine. For the other transporting systems, Se-Met and methionine revealed almost equal affinities. The apparent affinity for the Se-Met transport in OK cells (assessed via selenium content)

was equal to that measured in B^0AT1 expressing oocytes, suggesting that B^0AT1 is the major component for uptake into these cells. In Caco-2 cells, the affinity for Se-Met uptake was similar to that of $b^{0,+}$rBAT expressing oocytes, suggesting this system to mediate most of the Caco-2 uptake. Moreover, these data are in agreement with the results of Leblondel and McConnell et al. (Leblondel *et al.*, 2001; McConnell and Cho, 1965) who suggested a common transport mechanism for selenomethionine and methionine in Caco-2 cells and in everted intestinal sacs of hamsters.

Cysteine and methylselenocysteine

MSC and cysteine were transported by SIT1, $b^{0,+}$rBAT and B^0AT1. The highest affinity for MSC was observed with $b^{0,+}$rBAT, the lowest with B^0AT1. For all transporters, the K_m values of MSC were significantly lower than those of cysteine. The reason for this difference is most probably not the substitution of sulphur for selenium, but rather the methylation of the side chain (Fig. 20). This is in agreement with the data of Kowalczuk et al. who recently reported a higher affinity of methylated amino acids for SIT1 (Kowalczuk *et al.*, 2005). Selenocysteine could not be experimentally tested, because it is a highly redox active amino acid, which is easily converted to Se-Cys. The affinities for MSC in OK and Caco-2 cells were similar, but lower than observed in oocytes and were closer to the affinities measured in B^0AT1 expressing oocytes suggesting that B^0AT1 could be the major transport system for uptake of MSC in these cells.

Cystine and selenocystine

For cystine and Se-Cys only oocytes expressing $b^{0,+}$rBAT generated significant transport currents. The affinity for Se-Cys was significantly lower than that for cystine but nearly equal maximal transport currents were obtained for both, which were however around 50% lower than the maximal current evoked by leucine. Previous studies reported contradictory results: Wollffram et al. detected in porcine BBMV - in accordance with these present data - a competitive interaction of cystine and Se-Cys for transport (Wolffram *et al.*, 1989), whereas McConnell et al. failed to show cystine inhibition of transmucosal movement of Se-Cys at similar concentrations (McConnell and Cho, 1965). In OK cells, a lower affinity for Se-Cys than for transport via $b^{0,+}$rBAT in oocytes was detected. This could be due to a different membrane potential or a different lipid environment in the apical membrane as compared to oocytes. Another possibility may be a reduction of Se-Cys by extracellular enzymes to selenocysteine, which could make this compound also available for several other transport systems like B^0AT1 or SIT1. In Caco-2 cells, the affinity for Se-Cys was similar to that measured in $b^{0,+}$rBAT expressing oocytes.

Betaine and selenobetaine

In oocyte experiments, betaine and Se-Bet were transported by SIT1 and PAT1. In oocytes expressing SIT1, the affinity for Se-Bet was several times lower than that for betaine, in oocytes expressing PAT1, a very low affinity (K_m > 20 mM) for Se-Bet and a somewhat higher affinity for betaine were measured. Thus, for both transporters, the affinities for betaine were much higher and this may be due to the replacement of the nitrogen by selenium in Se-Bet. In OK cells, the kinetic measurements showed for Se-Bet a similar affinity constant as in SIT1 expressing oocytes, suggesting that SIT1 could be the major route of absorption. In Caco-2 cells, an affinity constant for Se-Bet could not be calculated and by using competition assays with L-proline, Se-Bet showed also a very low inhibition confirming the low affinity detected in PAT1 expressing oocytes. Due to its low affinity for both transporters SIT1 and PAT1, Se-Bet has obviously no important role in supplying organic selenium to the organism.

Apical reabsorption of dibasic amino acids and cystine in kidney proximal tubule is mainly mediated by the heteromeric amino acid antiporter $b^{0,+}$rBAT (Feliubadalo *et al.*, 1999). Using the oocyte system it was tested, whether elevation of the amino acid concentration on the cytosolic surface results in an enhanced exchange activity. The injection of leucine to reach cytosolic concentrations of about 20 mM resulted in an 4.8-fold increase of arginine-induced inward currents at +60 mV, whereas the injection of the same amount of arginine enhanced the leucine-induced outward current 1.5-fold, suggesting a lower concentration of leucine than that of arginine in the oocyte. The higher stimulating effect was probably caused by a bigger difference of intracellular levels of leucine after injection (Meier *et al.*, 2002). As Meier and coworkers showed (Meier *et al.*, 2002), intracellular concentrations of arginine, histidine and lysine are in a range of 250 – 300 pmol per oocyte, whereas the concentrations measured for leucine were negligible.

Intracellular amino acid levels

The reason for the decrease of intracellular taurine and glutamic acid levels in OK cells after exposure to Se-Met and MSC is not known, but a possible explanation could be that the seleno compounds may also play a role in cell volume homeostasis by acting as osmolytes or by affecting the status of the important cytosolic osmolytes taurine and glutamic acid. Why Se-Bet did not evoke similar changes as the other two compounds cannot be answered presently.

In conclusion, a low selenium availability could be caused not only by a low supply but also by an inadequate absorption of organic selenium compounds. As several diseases associated with amino acid transporter defects are known, like a malfunction of B^0AT1 in the case

of Hartnup disorder, the elucidation of the cellular pharmacokinetics is of great importance for further improvement of therapeutic facilities. This present analysis of epithelial transport of selenium-containing amino acids demonstrates that various distinct amino acid transporting systems are involved in the intestinal absorption and the renal reabsorption of these compounds. Based on the relatively high affinities of the organic selenium compounds for the B^0AT1 transport system, the similar affinities between oocytes and cell lines and the high uptake rates observed in both cell lines, the B^0AT1 system may dominate overall transport of selenoamino acids. Moreover, Se-Met and MSC seem to be the best organic selenium sources for efficiently supplying cells with organic selenium.

3 Cysteine and glycine and cysteinyl-glycine as cell-protectants against oxidative stress

3.1 Transport of dipeptides in mammals

The end products of intestinal protein digestion are represented by a mixture of free amino acids and small peptides produced by luminal and membrane-bound proteases and peptidases (Brandsch, 2006). Similarly in kidney, peptides are produced by break-down of filtered larger peptides and proteins. In addition, short chain peptides circulating in blood will be reabsorbed in the tubular cell by the apical peptide transporter (Ganapathy and Leibach, 1986; Liu *et al.*, 1995).

In mammals two different transport systems - PEPT1 and PEPT2 - have been identified. PEPT1 is primarily expressed in the small intestine and, to a much smaller extent, in the kidney showing highest expression level in the brush border membrane of renal proximal tubular cells (Lin *et al.*, 1999). Substrates for these transporters are small peptides consisting of two or three amino acids. Thereby, they contribute - together with amino acid transporters – to amino acid absorption, homeostasis and prevent in kidney a loss of these metabolites. Whether amino acid or peptide transporters are the major pathways for supplying the body with amino acid nitrogen is currently not known, but it is believed that the peptide transporters are more important (Leibach and Ganapathy, 1996). Intestinal and renal dipeptide uptake is driven by an inside directed H^+ gradient generated by a Na^+/H^+ exchanger in the apical membrane and therefore indirectly linked to the transmembrane Na^+ -gradient, which is required for uptake of glucose, many amino acids and vitamins.

For the dipeptide cysteinyl-glycine (CG), it was shown that it is a substrate of the peptide transporter PEPT2 and that it may serve as a good precursor for intracellular glutathione synthesis and protect neuronal cells against oxidative stress (Dringen *et al.*, 1999). Urinary CG is predominantly the product of extracellular degradation of glutathione. In the renal tubule, glutathione is cleaved by γ-glutamyl-transferase to CG and a γ-glutamic acid. CG is most likely reabsorbed by two routes: it can be taken up by peptide transporters or cleaved by membrane-bound peptidases to the constituent free amino acids taken up by amino acid transporters acting in parallel in the apical membrane of tubular epithelial cells. Frey et al. investigated metabolic alterations in PEPT2-knockout mice and showed a putative role of PEPT2 in metabolism of glutathione (Frey *et al.*, 2007). In PEPT2 knockout mice, they observed a highly increased renal excretion of CG and lower concentrations of cysteine (cys)

and glycine (gly) in kidney tissue, whereas tissue concentrations of glutathione were not changed.

Increased oxidative stress and a proinflammatory state are frequently found in patients with chronic renal failure (Cottone *et al.*, 2008), which is associated with risk factors such as hypertension, diabetes, dyslipidemia, obesity and the metabolic syndrome. Oxidative stress occurs when high levels of reactive oxygen species endogenously produced (ROS; H_2O_2, superoxide, hydroxyl radical) overwhelm the protection mechanism provided by cellular antioxidants such as glutathione (Jones, 2002). It is therefore considered that an adequate supply of the substrates cys, gly and glu for GSH synthesis may help protect renal functions in the disease states.

In the framework of the present thesis, it was assessed whether cells exposed to the free amino acids cys and gly as compared to the dipeptide CG show different levels of protection to oxidative stress artificially induced in cells. For this study section, the renal $LLCPK_1$ cell line that expresses a PEPT2 protein was used. In addition, OK cells that lack expression of a functional peptide transporter were transfected with a construct to obtain a functional PEPT2 with non-transfected cells serving as a control. The cells capacity to scavenge peroxides was tested by using tert-Butyl peroxide (BP) as the stress inducing agent.

3.2 Results

3.2.1 Reduction of organic peroxides by the dipeptide cys-gly and single amino acids inside $LLCPK_1$ cells

For assessing the effects of CG compared to the free amino acids gly and cys, $LLCPK_1$ cells were loaded with the peroxide-sensitive dye CM-H_2DCFDA and peroxide generation was induced by using BP.

Cells first were preincubated with gly and cys or CG for one hour followed by treatment with BP. In cells exposed to BP, fluorescence intensity increased over time (Fig. 31A). This increase was reduced by both, CG and the amino acids. However, CG showed the lowest effect on reduction of fluorescence, cys and gly the highest, whereas cysteine alone was as effective as the amino acid mixture.

The effects were also measured, when BP was applicated concomitant with CG and cys and gly (Fig. 31B). Here for gly and/or cys, the capacity to reduce peroxide-levels was slightly lower than after preincubation with the compounds. CG showed a lower capacity compared to the preincubation-experiment.

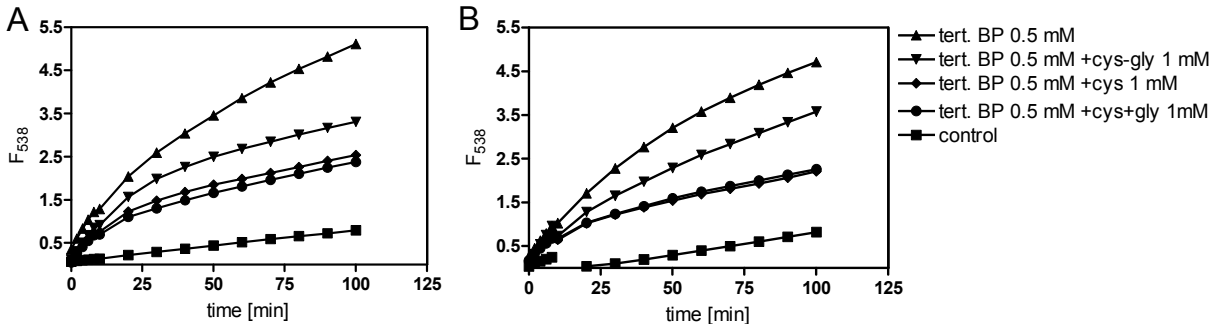

Fig. 31 - H_2DCFDA-fluorescence recorded in $LLCPK_1$ cells after treatment with tert. BP (0.5 mM) and cys and/or gly or cys-gly over 100 min. (A) Cells were preincubated with dye or amino acid(s)/dipeptides for 1h. (B) Cells were preincubated with dye, amino acids/dipeptides were simultaneously given with tert. BP (0.5 mM). Data are presented as means (n = 4).

3.2.2 Overexpression of the peptide transporter PEPT2 in OK cells

CG can be taken up into cells by the renal peptide transporter PEPT2 or after cleavage by peptidases as free amino acids by transporters such as $b^{0,+}$rBAT and B^0AT1. To decipher the role of PEPT2 in mediating a higher antioxidative capacity, it was investigated, if the effects of CG on scavenging peroxides are higher in cells expressing PEPT2 compared to cells lacking this transporter.

For this experiment, the OK cell line, lacking this transporter, was transfected with a PEPT2 vector construct as described in the Methods' section. The pept2 gene was C-terminally fused with a pH-sensitive green-fluorescent protein (GFP) named "pHluorin". The overexpression of PEPT2 was proven by uptake measurements and fluorescence microscopy with radio- or fluorescent-labelled substrates. Both, the fluorescent-labelled dipeptide ß-Ala-Lys-AMCA and the [^{14}C]-labelled dipeptide Gly-Sar as well served as good substrates, indicating a functional expression of PEPT2 in the apical membrane of the OK cells (Fig. 32A, B). Furthermore, the green-dotted coloration shows the microvilli on the surface of the cells (Fig. 32C). Uptake of both dipeptides was inhibited by adding an excess amount of non-labelled Gly-Gln (Fig. 32A).

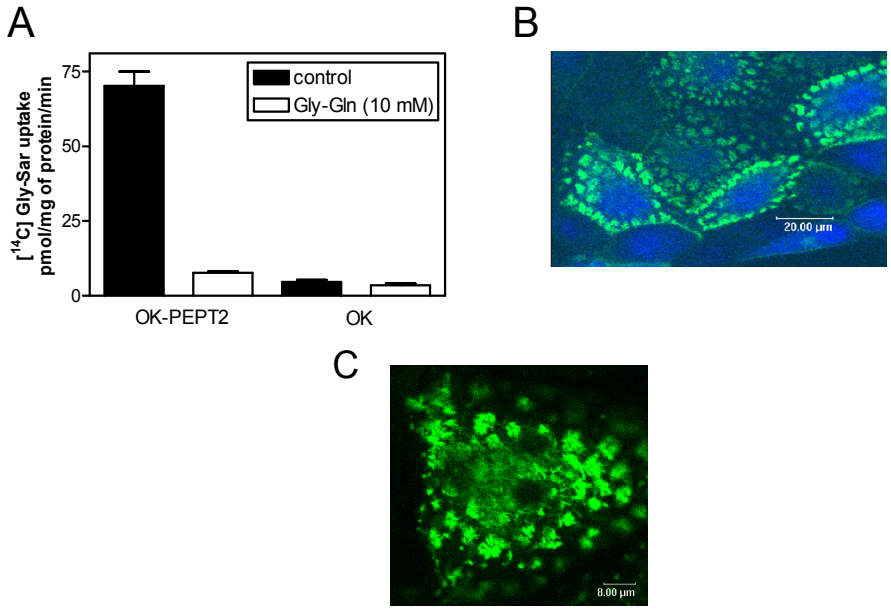

Fig. 32 - Uptake of labelled dipeptides in OK and OK-PEPT2 cells. (A) Uptake of [^{14}C]Gly-Sar (17.5 µM) was measured for 10 min in the presence or absence of Gly-Gln (10 mM) (n = 4-6). (B) Uptake of ß-Ala-Lys-AMCA (50 µM) was measured for 30 min in OK-PEPT2 cells. (C) pH-luorin-fluorescence in the apical membrane of OK-PEPT2 cells.

3.2.3 Reduction of organic peroxides by the dipeptide cys-gly and the corresponding free amino acid in OK cells

First, both cell lines were preincubated with CG for one hour. After loading with peroxides, fluorescence intensity increased in both cell lines (Fig. 33A,B). However, the increase in OK-PEPT2 cells was slightly, but not significantly higher. In OK cells pretreated with CG, increase was markedly reduced just like in OK-PEPT2 cells.

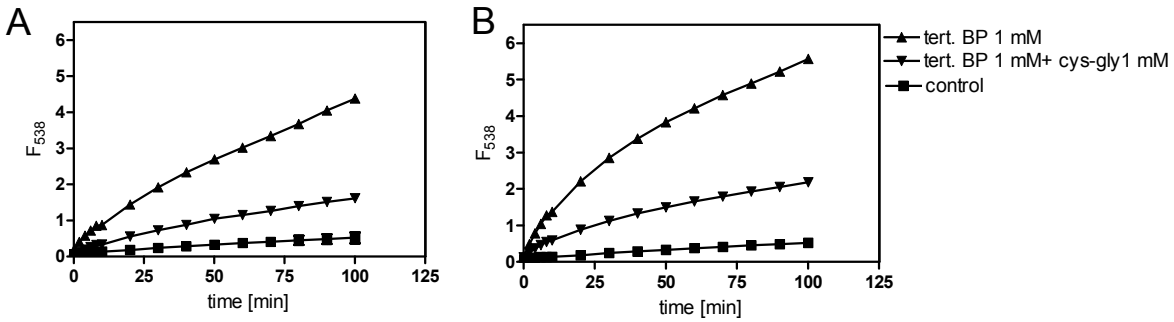

Fig. 33 - H_2DCFDA-fluorescence inside OK (A) and OK-PEPT2 (B) cells after treatment with tert. BP (1 mM) over 100 min. Cells were preincubated with dye and cys-gly for 1h. Data are presented as means (n = 4).

Furthermore, both cell lines were exposed to different concentrations of peroxides concomitant with CG (Fig. 34). Interestingly, OK-PEPT2 cells showed a higher increase of fluorescence than non-transfected cells. At a concentration of 0.5 mM, CG could only slightly reduce the increased fluorescence by BP in OK cells, whereas in OK-PEPT2 cells fluorescence was markedly reduced. At the higher concentration of 1 mM, CG could markedly reduce fluorescence in both cell lines. However, the reduction of peroxide-levels was more pronounced in PEPT2-expressing cells, but the differences between the cell lines were not altered compared to the exposure to the lower concentration of BP.

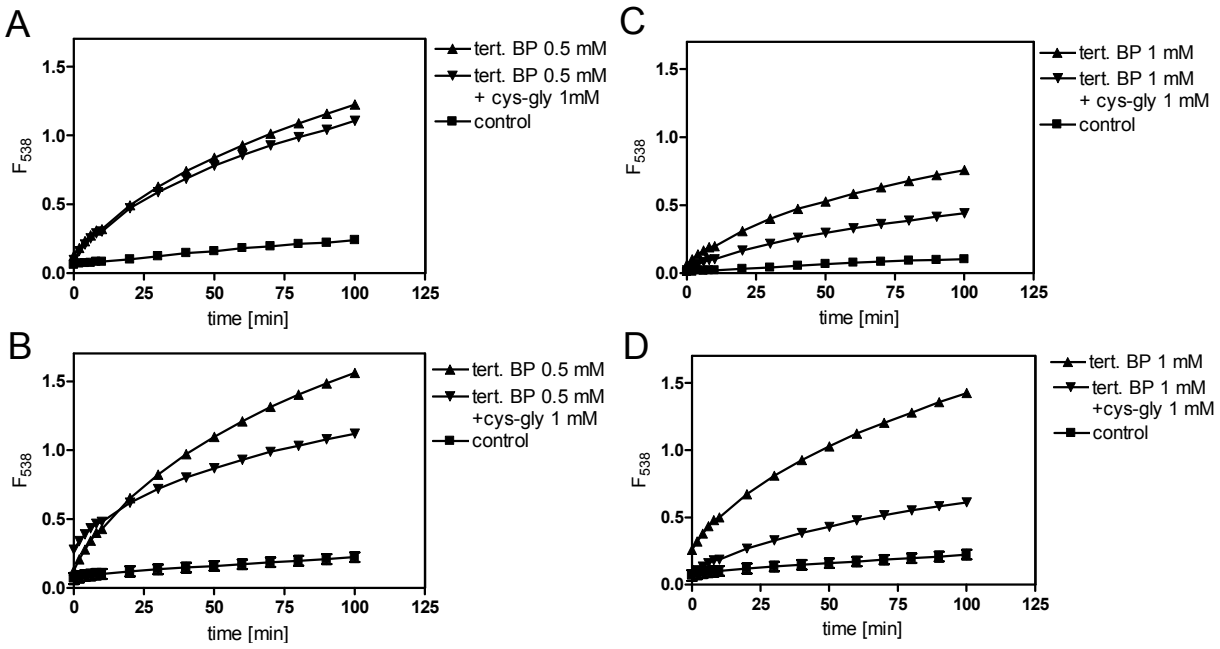

Fig. 34 - H_2DCFDA-fluorescence inside OK and OK-PEPT2 cells after treatment with tert. BP (0.5 mM or 1 mM) and cys-gly (1 mM) over 100 min, respectively. (A) OK cells were preincubated with dye, cys-gly was simultaneously given with tert. BP (0.5 mM). (B) OK-PEPT2 cells were preincubated with dye, cys-gly was simultaneously given with tert. BP (0.5 mM). (C) OK cells were preincubated with dye, cys-gly was simultaneously given with tert. BP (1 mM). (D) OK-PEPT2 cells were preincubated with dye, cys-gly was simultaneously given with tert. BP (1 mM). Data are presented as means ($n_{cys\text{-}gly}$ = 7-8, $n_{control}$ = 4).

3.3 Discussion

In this project part, the capacity of cysteinyl-glycine (CG) and the corresponding free amino acids to reduce oxidative stress induced by peroxides in $LLCPK_1$ cells was assessed. All tested compounds were capable to scavenge organic peroxides but free cysteine and glycine provided a stronger protection than CG. The effect of cysteine is explained by the antioxidative effect of its sulfhydryl group and its function as a component of glutathione. In many cell types, cysteine is the limiting factor for glutathione synthesis (Bannai and Tateishi, 1986).

The slightly higher protective effect of additional glycine may be due to an increased capacity to synthesize glutathione from these two amino acids. The lower capacity of CG to scavenge peroxides compared to the individual amino acids could be due to a higher transport capacity and number of amino acid transporters than peptide transporters in the apical membrane of the $LLCPK_1$ cells allowing more efficient delivery of protective amino acids. The observed higher activity to scavenge peroxides after a preincubation of cells as compared to a simultaneous exposure may also be explained by a higher synthesis capacity for glutathione and/or a higher amount of non-oxidised cysteine available.

A second series of experiments addressed the role of peptide transporters in cysteinyl-glycine (CG) handling in kidney. Here the effect of CG on scavenging of peroxides was compared in native OK cells and a cell line transfected with the PEPT2 transporter. The results showed interestingly a slightly higher increase of ROS fluorescence in OK-PEPT2 cells when the cells were exposured to BP. This may be caused by a more efficient permeation of either the dye into the OK-PEPT2 or that these cells have in total a lower antioxidative capacity. Since H_2DCFDA is an organic anion, its higher accumulation in the cells expressing PEPT2 seems feasible as changes in intra- and extracellular pH in the presence of the peptide transporter could alter its cellular accumulation. As far as intracellular glutathione levels in kidney cells are concerned, wildtype and PEPT2-knockout mice did not reveal any differences here suggesting that PEPT2 does not play an important role for GSH synthesis and maintenance, although the transporter-lacking animals had an increased renal loss of CG and cysteine and glycine (Frey *et al.*, 2007). Although only a negligible transport capacity for the reference substrate Gly-Sar was detectable in OK cells, they showed a high decrease of BP-induced fluorescence after preexposure to CG, which may result from a high activity of extracellular peptidases cleaving CG followed by uptake of the free amino acids and increased scavenging of peroxides. Both, OK and OK-PEPT2 cells showed a high antioxidative capacity against higher concentration of BP (1mM) when applied simultaneously with the dipeptide but there was an advantage of cells expressing PEPT2 in maintaining lower peroxide levels.

Taken together, the amino acids cysteine and glycine as well as the dipeptide CG when taken up into renal cells allow an efficient scavenging of organic peroxides. Furthermore, cells expressing the high-affinity peptide transporter showed a better - CG-mediated - defense against peroxide driven ROS generation. Thus, PEPT2 may play a significant role in efficient reabsorption of CG and to provide thiols as protectants that could help to delay progression of renal diseases associated with increased ROS production. In this context, it seems worth to mention that glycine is considered as well a renal protectant and is usually included to preserve isolated kidneys for transplantation and has proven to have cytoprotective effects in renal tissues, although this mechanism of action is not understood (Habib *et al.*, 2006; Tang *et al.*, 2006). To better understand the processes by which cysteine, glycine and cysteinl-glycine provide antioxidative potential to renal cells, the determination of intracellular glutathione-levels (reduced and oxidized) seems necessary. Additionally, the activity of extracellular peptidases and the alterations of intracellular cysteine and glycine levels in the renal cells after administration of the dipeptide CG should be determined.

4 Summary

Renal reabsorption of amino acids in the proximal tubule is achieved by a concerted action of several amino acid transporting systems. More than 95% of the amino acids from the ultrafiltrate are reabsorbed in the S1 – S3 segments of the proximal tubules (Broer *et al.*, 2005). Recently, a membrane transporter for the imino acid proline SIT1 (Sodium / Imino-acid Transporter, *SLC6A20*), a member of the Na^+- and Cl^--dependent neurotransmitter family, was identified as a candidate for the classical System IMINO in kidney and for hereditary iminoacidurias in humans (Kowalczuk *et al.*, 2005; Takanaga *et al.*, 2005b).

In exploring OK cells as a tool for characterizing imino acid transport, SIT1 expression in OK cells was studied by PCR and proline tracer uptake experiments were performed. By PCR, the mRNA of a homologue SIT1 gene from opossum could be amplified. Proline transport in OK monolayers was Na^+- and Cl^- -dependent, pH sensitive as transport activity decreased with lower pH values, showed very poor transport when Na^+ was replaced by Li^+ and showed a high affinity (K_m = 0.22 ± 0.08 mM), typical for the high-affinity SIT1 transporter. In competition assays, uptake of [^{3}H]L-proline was dose-dependently reduced by the non-labelled SIT1 substrates, e.g. hydroxyproline, N-methylproline, pipecolate and MeAIB, while typical PAT substrates, like taurine, γ-aminoisobutyric acid, cycloserine and vigabatrin, did not. Other hydrophobic amino acids such as leucine and phenylalanine are also substrates for SIT1 but display markedly reduced affinities. L-stereosiomers are preferred over D-isomers by the L-proline transport system in OK cells. A PAT-like proton-dependent uptake of proline could not be detected in OK cells, although mRNA of PAT1 and PAT2 was found. In conclusion, a SIT1 transport protein is expressed in OK cells and L-proline transport shows all properties of system IMINO in kidney, which makes these cells an appropriate model to investigate the mechanisms underlying hereditary iminoacidurias.

For a further characterization of proline transport, OK cells were exposed to amino acid deprivation. When OK cells were submitted for 8 h to amino acid deprivation, uptake of L-proline increased 3-fold with kinetic analysis 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 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. Up-regulation caused by amino acid deprivation was prevented in the presence of the protein biosynthesis inhibitors actinomycin, cycloheximide and by supplementing media with L-proline or mixtures of other amino acids during the deprivation period as well. The low- and the high-affinity proline transporting systems were sensitive to inhibitors of JNK and PI-3 kinases, whereas a GSK-3 inhibitor affected only the transport system that mediates uptake

under amino acid deprivation. Ion-replacement studies and experiments assessing substrate specificities for both systems provided strong evidence for SNAT2 as the responsible transporter mediating increased proline influx under amino acid deprivation. In support, mRNA levels of SNAT2 were increased 2 to 3-fold. Thus, it may be concluded that amino acid deprivation leads to *de novo* synthesis of a second L-proline transport system in OK cells that has a low affinity but high capacity and may be identical with SNAT2.

Selenoamino acids are the main form of organic selenium derived from the diet. They are efficiently absorbed in the intestine and reabsorbed in kidney, but the transporter proteins that mediate their cellular uptake have not yet been identified. The transport pathways of selenoamino acids and derivatives, including selenomethionine, methylselenocysteine, selenocystine, selenobetaine and selenocystamine are described for the first time in this thesis. Transport studies employed the *Xenopus laevis* oocyte system expressing the amino acid transporters SIT1, $b^{0,+}$rBAT, B^0AT1 or PAT1 and intestinal Caco-2 and renal OK cell lines that possess a multitude of amino acid transporters. The results suggest that the major route for the uptake of selenomethionine is the system $b^{0,+}$rBAT in Caco-2 cells and B^0AT1 in OK cells. Affinities of selenomethionine or methionine for these transporters did not differ, but for SIT1 selenomethionine shows a higher affinity than methionine. Methylselenocysteine displayed a higher affinity than cysteine for all transporters tested and in both OK and Caco-2 cells system B^0AT1 seems to be the primary uptake route. Selenocystine is taken up well by the $b^{0,+}$rBAT system, while selenobetaine is a low-affinity substrate only for SIT1 and PAT1. Selenocystamine was not transported by any of the transport systems investigated. When cells were exposed to selenoamino acids, intracellular selenium levels in OK cells considerably exceeded those in Caco-2 cells, indicating effective renal reabsorption capacity. In conclusion, selenoamino acids but not the seleno-derivatives selenobetaine and selenocystamine, are effectively transported by various intestinal and renal amino acid transporters and are thus available for selenium metabolism and therapeutic approaches.

PEPT2 functions as a high-affinity transporter for small peptides consisting of two or three amino acids, predominantly expressed in the renal proximal tubule. As Frey et al. demonstrated, PEPT2 is the major system for reabsorption of the dipeptide cysteinyl-glycine originating from GSH break-down (Frey *et al.*, 2007). Furthermore, Dringen et al. could demonstrate that cysteinyl-glycine is a good precursor of glutathione in neuronal cells und so offers a better protection against oxidative stress (Dringen *et al.*, 1999). In some renal diseases, e.g. chronic renal failure, oxidative stress occurs and the maintenance of glutathione-levels plays a crucial role to prevent further progression of the disease. It was therefore assessed in renal cells whether the free amino acids cysteine and glycine compared to cysteinyl-glycine

are able to scavenge organic peroxides and whether CG provides an advantage over the free amio acids. All tested compounds could reduce ROS-levels but the free amino acids showed the strongest and cysteinyl-glycine the lowest effect in the $LLCPK_1$ cell line and this may be related to a much higher capacity of uptake of free amino acids over dipeptides in this renal cell model. To address this in a second model, the effects of cysteinyl-glycine were also assessed in OK cells transfected to express higher levels of PEPT2 as compared to native OK cells that lack essentially this transporter. The reduction of peroxide-levels in the cells was more pronounced when cells provided with CG expressed PEPT2, both after preincubation or with a concomitant exposure to peroxides. Surprisingly, PEPT2-expressing cells exhibited in general higher peroxide-levels and showed an increased capacity to retain the fluorescent probe, which may be related to the function of the peptide transporter in pH homoeostasis. However, a putative role of PEPT2 in cysteinyl-glycine mediated protection against oxidative stress induced by organic peroxides is shown and the delivery of cell protectants via PEPT2 could help to delay the progression of renal diseases associated with an increased burden of reactive oxygen species.

Taken together, in the thesis work described here, selected amino acid and peptide transport processes in renal cell models have been characterized and this relates to transport of imino acids as well as sulphur and selenium containing amino acids and peptides. It was specifically shown that renal cells express at high levels a prototypical SIT1 transporter for uptake of imino acids and related compounds. In cells undergoing amino acid depletion, cells adapt quickly to an increased amino acid demand by enhanced expression of other transporters such as SNAT2. Moreover, for the first time the transport of selenoamino acids and derivatives was characterized on basis of distinct amino acid transporters found in epithelial cells by combining electrophysiological measurements with tracer flux studies and chemical analysis of the selenium and the amino acids in the cells. And finally, the specific role of cysteine and glycine or the corresponding dipeptide cysteinyl-glycine and their uptake via amino acid and peptide transporters into renal cells was assessed in context of their cell protectant activity to scavenge reactive peroxides that can damage the kidney.

5 Zusammenfassung

Die Reabsorption von Aminosäuren im proximalen Tubulus der Niere wird durch die Aktivität einer Vielzahl von Aminosäuretransportsystemen vermittelt. Mehr als 95% der Aminosäuren des Primärfiltrats werden in den S1 – S3 Segmenten des proximalen Tubulus reabsorbiert (Broer *et al.*, 2005). Der Membrantransporter für die Iminosäure Prolin, SIT1 (Sodium / Imino-acid Transporter, *SLC6A20*), als Kandidat für das klassische System „IMINO“ ist erst kürzlich identifiziert worden.

In OK Zellen als Modellsystem zur Charakterisierung renaler Aminosäuretransportprozesse wurde die SIT1 Expression mittels PCR bestimmt und die Prolinaufnahme ermittelt. Mittels PCR konnte die mRNA eines homologen SIT1 Gens aus Opossum amplifiziert werden. Die Aufnahme von Prolin in die Zellen war Na^+- und Cl^- -abhängig sowie pH-sensitiv und zeigte nur eine geringe Transportrate wenn Na^+ durch Li^+ ersetzt wurde. Eine beobachtete hohe Affinität (K_m = 0.22 ± 0.08 mM) für Prolin ist charakteristisch für das SIT1-Transportsystem. In Kompetitionsstudien wurde die Aufnahme von [³H]L-Prolin durch nicht markierte SIT1-Substrate, wie Hydroxyprolin, N-Methylprolin, Pipecolat und MeAIB, deutlich reduziert, während typische Substrate des protonen-abhängigen Systems PAT wie Taurin, γ-Aminoisobuttersäure, Cycloserin und Vigabatrin die Aufnahme nicht reduzierten. Andere hydrophobe Aminosäuren wie Leucin und Phenylalanin erwiesen sich auch als SIT1-Substrate, zeigten aber deutlich niedrigere Affinitäten. L-Stereoisomere werden D- Stereoisomeren gegenüber bevorzugt, eine protonenabhängige, für PAT charakteristische Aufnahme von Prolin konnte nicht nachgewiesen werden, obwohl die mRNAs von PAT1 und PAT2 nachgewiesen wurden. Folglich wird ein SIT1 Transportprotein in OK Zellen exprimiert und zeigt alle Eigenschaften des Systems „IMINO“ in der Niere, das diese Zelllinie zu einem geeigneten Modell zur Untersuchung der Grundlagen erblicher Iminoglyzinurie macht.

Zur weiteren Charakterisierung des Prolintransportes wurden OK Zellen einem 8-stündigen Aminosäureentzug ausgesetzt. Dabei zeigte sich, dass die Prolinaufnahme auf das dreifache des Ausgangswertes gesteigert werden kann und nun in der Kinetik durch ein niedrig-affines System mit einem K_m-Wert von 4.4 ± 0.6 mM und einer V_{max} von 10.2 ± 0.6 nmol/mg Protein/min - neben dem hochaffinen System – dominiert. Diese Adaptation in der Transportleistung wurde in Anwesenheit der Proteinbiosyntheseinhibitoren Actinomycin und Cycloheximid verhindert, was impliziert, dass hier eine Neusynthese von Transportern adaptativ erfolgt. Der Zusatz von Prolin, aber auch mehrerer anderer Aminosäuren konnte ebenso die Adaptation verhindern, was auf einen spezifischen Signalweg der Steuerung hinweist. Das niedrig- und das hochaffine, Prolin-transportierende System zeigten sich sensitiv gegenüber Inhibitoren von JNK und PI-3 Kinasen. Ein GSK-3 Inhibitor hingegen zeigte nur beim adaptativen

System eine Wirksamkeit. Sowohl Untersuchungen zur Ionenabhängigkeit als auch Substratspezifität für beide Systeme lassen vermuten, dass SNAT2 jenes Transportsystem ist, das unter Aminosäureentzug den gesteigerten Transport vermittelte. Auch die mRNA-Spiegel von SNAT2 waren um das 2- bis 3-fache erhöht. Aminosäureentzug in renalen OK Zellen führt zur *de novo* Synthese eines zweiten Prolin-transportierenden Systems mit niedriger Affinität aber hoher Transportkapazität und ist wahrscheinlich dem SNAT2-Transporter zuzuschreiben.

Selenoaminosäuren sind die Hauptquellen organischen Selens aus der Nahrung. Sie werden effizient im Dünndarm absorbiert und in der Niere reabsorbiert, aber die Transportproteine, die ihre zelluläre Aufnahme vermitteln, wurden bisher nicht identifiziert. Die Transportwege von Selenoaminosäuren und ihrer Derivate, d.h. Selenomethionin, Methylselenocystein, Selenocystin, Selenobetain und Selenocystamin wurden in dieser Arbeit zum ersten Mal beschrieben. Für die Transportstudien wurden das *Xenopus laevis* Oozyten System, das die Aminosäuretransporter SIT1, $b^{0,+}$rBAT, B^0AT1 oder PAT1 exprimierte, sowie die intestinale Caco-2 Zelllinie und die renale OK Zelllinie verwendet, die eine Vielzahl von Aminosäuretransportsystemen in ihrer apikalen Membran exprimieren. Die Ergebnisse legen als Haupttransportweg für Selenomethionin in Caco-2 Zellen das $b^{0,+}$rBAT System und B^0AT1 in OK Zellen nahe. Die Affinitäten von Selenomethionin und Methionin für diese Transporter unterscheiden sich nicht signifikant, aber für SIT1 zeigt Selenomethionin eine höhere Affinität als für Methionin. Methylselenocystein zeigte eine höhere Affinität als Cystein für alle untersuchten Transporter und in beiden Zelllinien scheint B^0AT1 der Haupttransportweg zu sein. Selenocystin wird vom $b^{0,+}$rBAT System gut aufgenommen, während Selenobetain nur ein niedrigaffines Substrat für SIT1 und PAT1 ist. Selenocystamin wurde von keinem der untersuchten Transportsysteme aufgenommen. Die Inkubation beider Zelllinien mit Selenoaminosäuren zeigte deutlich höhere intrazelluläre Selenspiegel in OK als in Caco-2 Zellen, was für eine hohe renale Reabsorptionskapazität spricht. Alle Selenoaminosäuren mit Ausnahme der Derivate Selenobetain und Selenocystamin werden effizient von den Aminosäuretransportern in die Zellen aufgenommen und stehen damit für den Selenstoffwechsel zur Verfügung. Aus den Studien lassen sich therapeutische Anwendungen zur Verbesserung der Bioverfügbarkeit der zellulären Selenversorgung ableiten.

PEPT2 ist ein hochaffines Transportsystem für Di- und Tripeptide und wird hauptsächlich im proximalen Tubulus der Niere exprimiert. Wie Frey et al. zeigen konnten, ist PEPT2 das System in der apikalen Membran von renalen Epithelzellen, das das Dipeptid Cysteinyl-glyzin aus dem extrazellulären Glutathionabbau effizient in die Zelle aufnimmt (Frey *et al.*, 2007). Darüber hinaus konnten Dringen et al. zeigen, dass Cysteinyl-glyzin eine gute Vorstufe für

die Glutathion-Synthese in neuronalen Zellen darstellt und so einen besseren Schutz gegenüber oxidativen Stress vermittelt. Bei einigen Nierenerkrankungen, so auch bei chronischer Niereninsuffizienz tritt vermehrt oxidativer Stress auf und die Aufrechterhaltung des Glutathionspiegels ist elementar, um einem weiteren Fortschreiten der Krankheit entgegen zu wirken. Es wurde daher geprüft, inwieweit Cysteinyl-glyzin und die Aminosäuren Cystein und Glyzin als protektiv wirkende Agentien einer Peroxid-induzierten ROS-Bildung entgegen wirken können.

In renalen $LLCPK_1$ Zellen wurde dazu die Kapazität, organische Peroxide abzufangen, mit Glyzin und Cystein im Vergleich zum Dipeptid Cysteinylglyzin untersucht. Die einzelnen Aminosäuren zeigten dabei den stärksten, Cysteinyl-Glyzin den schwächsten Effekt. Darüber hinaus wurden die Effekte von Cysteinyl-Glyzin auf die antioxidative Kapazität in OK Zelllinien verglichen, die einerseits nativ keinen Peptidtransporter exprimieren und andererseits nach Transfektion mit PEPT2 eine recht hohe Transportkapazität besitzen. Die Reduktion der Peroxidspiegel war in PEPT2 exprimierenden Zellen sowohl nach Vorinkubation als auch bei gleichzeitiger Gabe mit den Peroxiden stärker ausgeprägt, wobei interessanterweise PEPT2 exprimierende Zellen insgesamt höhere Peroxidspiegel aufwiesen. Die mögliche Rolle von PEPT2 in der durch Cysteinyl-Glyzin vermittelten Schutzwirkung gegen oxidativen Stress – ausgelöst durch organische Peroxide – wurde somit gezeigt. Die PEPT2 Funktion in der Niere mag somit eine Protektion vor Gewebeschädigungen darstellen und einem Fortschreiten von Nierenerkrankungen, die mit einer erhöhten Belastung durch reaktive Sauerstoffspezies einhergehen, entgegen wirken können.

6 Materials

6.1 Equipment

Besides common laboratory equipment the following specific tools were used: Biophotometer (Eppendorf, Hamburg, Germany), Confocal laser-scanning microscope TCS SP2, including an inverted fluorescence microscope (Leica, Wetzlar, Germany), PCR Sprinter, Fluoroskan Ascent fluorescence multiwell plate reader and Multiskan Ascent multiwell-plate photometer (both from Labsystems, Bornheim-Hersel, Germany), atomic absorption spectrophotometer model Analyst 300 (PerkinElmer, Ueberlingen, Germany), an autosampler HTC PAL (CTC Analytics AG, Zwingen, Switzerland), Lightcycler (Roche, Mannheim, Germany), Apt line CB incubator (Binder, Tuttlingen, Germany), Heraeus KS benches (Kendro, Langenselbold, Germany), liquid scintillation counter (1450 MicroBeta TriLux, PerkinElmer, USA), Agilent 1200 HPLC system (Waldbronn, Germany).

6.2 Biochemicals and consumables

Unless otherwise specified all chemicals used (pro analysis quality) were from Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Plastic ware for cell culture was obtained from Renner (Dannstadt, Germany). Media and supplements and other reagents for cell culture were obtained from PAA (Coelbe, Germany) as well. Restriction endonucleases, nucleotides and DNA-markers were from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Beverly, MA, USA). Primers were from MWG (Ebersberg, Germany). RNAWIZ, formaldehyde and the Retroscript-Kit were purchased from Ambion (Kassel, Germany). Vigabatrin was from Tocris (Ellisville, USA). All radiochemicals (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).

Opossum kidney (OK) cells were kindly provided by H. Murer (University of Zurich, Switzerland) and were used between passage 40 and 60, the Caco-2 cell line was obtained from the German National Resource Centre for Biological Material (Braunschweig, Germany) and was used between passage 24 and 54. LLCPK$_1$ cell line was obtained from American Type Culture Collection Rockville, MD, USA) and used between passage 30 and 33.

Female *X. laevis* oocytes were purchased from African Xenopus facility (Knysna, RSA). The mouse B^{0}AT1-KSM plasmid (Camargo *et al.*, 2005), the mouse mb$^{0,+}$AT-psd5easy plasmid (Franca *et al.*, 2005), the human SPORT1-hrBAT: Acc.: L11696 (Furriols *et al.*, 1993) plasmid were kindly provided by F. Verrey (University of Zurich, Switzerland) and the mouse

mXT3s1 plasmid was kindly provided by S. Bröer (School of Biochemistry and Molecular Biology, Canberra, Australia).

6.3 Composition of solutions, buffers and gels

DEPC-water
0.1% DEPC
double distilled water

10x MOPS-buffer
0.2 M MOPS
0.05 M Sodium-acetate
0.1 M EDTA
DEPC-water
pH-value was adjusted at 7 with NaOH

TAE-buffer
42 g Tris
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA-solution

Sample-buffer for DNA gels (10-fold)
50% glycerine
10x TAE-buffer
0.02% bromohenolblue

RNA-gel (1% agarose)
0.8 g agarose
65 ml DEPC-water
8 ml 10x MOPS-buffer
6.6 ml 37% formaldehyde

DNA-gel (1.5% agarose)
1.1 g agarose
80 ml TAE-buffer
5 µl ethidiumbromide (conc.10 mg/ml)

RNA running-buffer
50 ml 10x MOPS-buffer
450 ml DEPC-water

DNA running-buffer
50 ml TAE-buffer
450 ml DEPC-water

Taq-buffer
10 ml 1M KCl
2 ml 1M Tris
0.5 ml 10% Tween
0.5 ml 10% Nonidet
0.5 ml 10 mg/ml BSA
0.4 ml 1M $MgCl_2$

phosphate-buffered saline (PBS), pH 7.4
136 mM NaCl
2.7 mM KCl
8.1 mM Na_2HPO4
1.5 mM KH_2PO4
pH-value was adjusted with Na_2HPO4

Igepal-lysis-buffer, pH 8.0
140 mM NaCl
1.5 mM $MgSO_4 \cdot 7\ H_2O$

modified Barth solution, pH 7.5
96 mM NaCl
2 mM KCl

0.5% Igepal CA-630
50 mM Tris
pH-value was adjusted with HCl

1.8 mM $CaCl_2$
2 mM $MgCl_2$
0.33 mM $Ca(NO_3)_2$
2.4 mM $NaHCO_3$
5 mM MES
pH-value was adjusted with Tris

uptake buffer for oocytes (pH 7.5/ 6.5)

88 mM NaCl
1 mM KCl
0.41 mM $CaCl_2$
0.82 mM $MgSO_4$
1 mM $CaCl_2$
0.33 mM $Ca(NO_3)_2$
2.4 mM $NaHCO_3$
10 mM HEPES pH 7.5 or 10 mM MES pH 6.5 for mPAT1 expressing oocytes
pH-value was adjusted with Tris

For uptake experiments in cells buffers containing (in mM) 140 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.0 $MgSO_4$, 5 glucose and 25 HEPES/Tris (pH 7.4) or 25 MES/Tris (pH 6.5 and pH 6.0) were used. For experiments without Na^+, NaCl was replaced by choline-Cl, for experiments without Cl^-, NaCl and KCl as well as $CaCl_2$ were replaced by Na-, K- and Ca-D-gluconate.
For testing Li^+-tolerance, NaCl was replaced by LiCl.

7 Methods

7.1 Culture of OK, OK-PEPT2, LLCPK$_1$ and Caco-2 cells

Cell culture work was performed under sterile conditions and media as well as buffers were prewarmed for 37°C before use. OK and OK-PEPT2 cells were cultured and passaged in DMEM/F-12 supplemented with 10% fetal calf serum and 1% Penicillin/ 1% Streptomycin, OK-PEPT2 additionally with G 418 (0.5 mg/ml).

The LLCPK$_1$ cell line was cultured and passaged in DMEM supplemented with 10% fetal calf serum, 1% Penicillin/Streptomycin, 2 mM glutamine, 1% MEM non-essential amino acids, 10 mM HEPES and 1 mM sodium pyruvate.

Caco-2 cells were cultured in medium consisting of Minimal Essential Medium (MEM) with Earle's salts and 2 mM L-glutamine, supplemented with 10% fetal calf serum, 1% Penicillin/ 1% Streptomycin and 0.1 mM MEM non-essential amino acids. After trypsinization they were additionally centrifuged at 1200 rpm for 3 min, the supernatant was discarded and the cells were resolved in 15 ml medium.

All 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 2.5 * 10^6/ 75 cm² flask, 0.5 * 10^6 /well of a 6-well-plate and 0.1* 10^6 /well of a 24-well-plate. Well-plates for seeding LLCPK$_1$ cells were additionally treated with collagen A (Biochrom, Berlin, Germany).

7.2 Transfections

7.2.1 Construction of a rPEPT2-pHluorin expression plasmid

The rpept2 gene was cloned from the pSPORT-rPEPT2 vector construct by PCR with primers (5'-3') CCAAGAAGACAAAGCTCTCACCGGTCCAGGACTCTGCCCTG and CAGGGCAGAGTCCTGGGACCGGTGAGAGCTTTGTCTTTGTCTTCTTGG. The PCR products were digested with AgeI/NotI and ligated into pEGFP-N1 (BD Bioscience, Heidelberg, Germany) where the EGFP was replaced by the pH-sensitive GFP "pHluorin".

7.2.2 Stable transfection of OK cells

OK cells were grown on 6-well-plates until they reached 50% confluency. Each well was transfected with 2 µg of plasmid DNA with the transfection reagent jetPEI (Biomol, Hamburg, Germany) according to the manufacturer's protocol. After two days of incubation, cells from each well were trypsinized, diluted 1:150 and 1:200 in fresh medium and seeded on 6-well-plates. After selection with G 418 (0.7 mg/ml) for two weeks, clones were picked and set on

96-well-plates. Clones were grown on 96-/24-/12- and 6-wells and passaged until they reached 80% confluency. PEPT2 expression of the clones was detected after excitation at 488 nm and emissions of 520-580 nm using a confocal laser scanning microscope.

7.3 Detection of peroxides

For detection of peroxides inside the cells, they were seeded as described before on 24-well-plates and OK as well as OK-PEPT2 cells, postconfluently grown for two days, $LLCPK_1$ cells postconfluently grown for ten days, were loaded with 5 or 10 µM of the peroxide-sensitive dye CM-H_2DCFDA (Molecular Probes, Leiden, Netherlands) for 1 h in buffer pH 7.4.
Subsequently to the loading with the fluorophor, cells were washed with buffer pH 7.4. Peroxide-generation inside the cells was achieved by the addition of 0.5 mM or 1 mM tertiary butylperoxide. The amount of peroxides inside the cells was followed over 100 min by measuring the fluorescence of H_2DCFDA at 538 nm after excitation at 485 nm using the fluorescence microtiter plate reader.

7.4 Amino acid deprivation

For amino acid-free incubation cell monolayers were washed once in buffer pH 7.4 and incubated in the same solution for the indicated periods.

7.5 Transport studies

7.5.1 Amino acid uptake assay

OK cells were grown on 6-well-plates and uptake assays were performed on the second day after cells reached confluency. If not indicated otherwise, buffer pH 7.4 was used. Immediately before the measurements, cell monolayers were washed once with the indicated buffer. 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. Other amino acids and potential substrates are added in excess amount as indicated. Cell monolayers were incubated for 6 min at room temperature and uptake was terminated by the removal of the test solution followed by three times washing with ice-cold buffer. Afterwards, the cells were solubilized with 1 ml Igepal-Lysis-buffer and radioactivity was determined by liquid scintillation counting.

Solutions containing actinomycin D, cycloheximide, SB-415286, wortmannin or JNK inhibitor II also contained 0.5 – 1% dimethylsulfoxide, equimolar controls were used. Further variations of this standard assay are given in the text.

7.5.2 Dipeptide uptake assay

The uptake assay was modified compared to the description in 7.5.1: the concentration of the radiolabelled substrate [^{14}C]-Gly-Sar (57 mCi/mmol, Amersham, Buckinghamshire, UK) was 17.5 µM, the uptake time was 10 min. Binding of tracer to the cells was determined as the residual radioactivity associated with the cells in the presence of excess non-labelled (10 mM) Gly-Gln.

For uptake assays with ß-Ala-Lys-AMCA (Amersham, Buckinghamshire, UK; 50 µM), the cells were grown on coverslips inserted into the six-well plates. Incubation with ß-Ala-Lys-AMCA was performed as described above for the radiolabelled peptide, the incubation time was extended to 30 min and the volume of the test solution was 0.5 ml at 37°C. After the cells were washed with ice-cold buffer, they were fixed for 15 min with 4% paraformaldehyde in PBS at room temperature. The coverslips were taken from the wells and put on slides. Specific uptake of ß-Ala-Lys-AMCA was detected after excitation with the UV-laser at emissions of 440-480 nm by using a confocal laser scanning microscope.

7.5.3 Xenopus laevis oocytes expressing mB^{0}AT1, mb$^{0,+}$hrBAT, mSIT1 and mPAT1

The oocyte from the South African clawed frog Xenopus laevis is an often used functional expression system. They have been exploited for the study of numerous aspects of ion channel function and regulation, such as the properties of several endogenous voltage-dependent channels and the involvement of second messengers in mediation of neurotransmitter-evoked membrane responses. In addition, injection of these cells with exogenous messenger RNA results in production and functional expression of foreign membranal proteins, including various voltage- and neurotransmitter-operated ion channels originating from brain, heart and other excitable tissues.

Xenopus laevis maintenance and oocyte harvest procedures were approved by the local authority for animal care in research (Regierung von Oberbayern, approval nr. 211-2531.3-9/99). Surgically removed oocytes were separated by collagenase treatment and handled as described previously (Boll et al., 1996). Individual oocytes were microinjected with 27 nl of RNA solution containing 65 ng of B^{0}AT1 cRNA, with 27 nl of RNA solution containing 38 ng of mb$^{0,+}$hrBAT cRNA, with 17 nl containing 25 ng of mPAT1 cRNA or with 27 nl containing 40

ng of mSIT1 cRNA into the cytoplasm of the oocyte. All electrophysiological measurements were performed after 3–6 days of incubation of oocytes in the modified Barth solution.

7.5.4 Electrophysiology

TEVC experiments were performed as described previously (Boll *et al.*, 2002; Wagner *et al.*, 2000). Briefly, the oocyte was placed in an open chamber and continuously superfused with uptake buffer in the absence or presence of the substances studied. Oocytes expressing B^0AT1, SIT1 or PAT1, were voltage-clamped at −60 mV using a TEC-05 amplifier (npi Electronic, Tamm, Germany). Oocytes expressing $b^{0,+}$rBAT, were voltage-clamped at -20 mV, since this transporter functions as an exchanger and the application of uncharged substrates on the extracellular surface resulted in the flow of positively charged amino acids out of the oocyte. Current–voltage (*I*–*V*) relationships were measured using short (100 ms) pulses separated by 200 ms pauses in the potential range −160 to +80 mV with 20 mV steps. *I*–*V* measurements were made immediately before and 20–30 s after substrate application, when current flow reached steady state. The currents at a given membrane potential were calculated as the difference between the currents measured in the presence and the absence of substrate.

7.5.5 Determination of protein concentration

Protein concentration was measured according to the procedure of Bradford (Bradford, 1976) in parallel samples not subjected to the transport assay.

7.6 Preparation of RNA

Total RNA of OK cells was isolated with RNAWIZ. Cells of a 75 cm² flask were scraped with 6 ml of RNAWIZ. After aliquoting in 1.5 ml-tubes and incubation at room temperature for 5 min, 200 µl of chloroform were added, the solution was shaken and incubated for 10 min at room temperature. Subsequently, the samples were centrifuged (11000 rpm; 4°C; 15 min) and the upper aqueous phase was given to a new 1.5 ml-tube and 500 µl DEPC-water and 1 ml of isopropanol were added and the sample was mixed. After incubation for 10 min at room temperature and centrifugation (11000 rpm; 4°C; 15 min), the supernatant was discarded, the pellet was washed with 1 ml 75% ethanol in DEPC-water (-20°C) and centrifuged (11000 rpm; 4°C; 5 min). The supernatant was discarded again and the pellet dried in a vacuum centrifuge for 2 min.

RNA was taken up and stored in RNase free water at –80°C. The yield was calculated based on the absorbance at 260 nm (absorption of 1.0 is equivalent to 40 µg/ml RNA). RNA integrity was verified by an absorption ratio (A_{260}/A_{280}) >1.8 and by denaturing agarose gel (1%)

electrophoresis at 80 V with 1x MOPS buffer. Intact total RNA preparations should appear as two bright bands with a ratio of intensities of the 28S and 18S rRNA of approximately 2:1.

7.7 Synthesis of cDNA and amplification of PCR fragments

First-strand cDNA was synthesized with the Retroscript-kit using random decamers as primers. The samples consisting of 3 µg RNA, 4 µl dNTPs, 2 µl random decamers and 15 µl water were denaturated at 75°C for 3 min and subsequently put on ice. After addition of 2 µl 10x RT-buffer, 1 µl RNAse-inhibitor (10 U/µl) und 2 µl MMLV-reverse transcriptase (100 U/µl) the reverse transcription was performed under following conditions: 45 min at 42°C, 15 min at 55°C and 10 min at 92°C.

After reverse transcription PCR samples consisting of 1 µl dNTPs, 2.5 µl Taq-buffer, 0.5 µl Taq-polymerase, 15 µl water and the primer pair were prepared.

Four gene-specific primer pairs for SIT1 and every two gene-specific primer pairs for PAT1 and PAT2 were used. DNA-sequence of SIT1 was obtained by a BLAST search on the Monodelphis domestica (opossum) genome at the Ensemble database (www.ensemble.org).

Four primer pairs (pp1: 5'GATCAATGCAGCTACCCAGAT3' bp 726-745 and 3'CATAGTCGTTGAATATGTCAAAC5' bp 1371-1393; pp2: 5'GCCAAGCAACAATTGCCAGAA3' bp 804-824 and 3'AAGGCCGATCACTGCCAAG5' bp 1668-1686; pp3 5'GATCAATGCAGCTACCCAGAT3' bp 726-745 and 3'AAGGCCGATCACTGCCAAG5' bp 1668-1686; pp4: 5'GCCAAGCAACAATTGCCAGAA3' bp 804-824 and 3'CATAGTCGTTGAATATGTCAAAC5' bp 1371-1393) were used to amplify gene-specific PCR products of SIT1 on first strand cDNA as template.

Following PAT1-specific primer pairs were used: pp1: 3'GGAAGTCAATGGAACAACACG5' bp 126-146 and 5'CTGCCAATGGCAAGTTTCTG3' bp 757-778;

pp2: 3'GCATTGTGGCTACACACTGT5' bp 263-282 and 5'GCACATGTTAGAGAGGCCAT3' bp 1163-1182

Following PAT2-specific primer pairs were used: pp1: 3'GCATTGTGGCAGTGCACTGTA5' Fa and 5'GTGCGGATGAACAAGTCTAC3' Ba

pp2: 3' GCATTGTGGCAGTGCACTGTA 5' Fa and 5'TGGAGGTATGATCAGAGCCA3' Bb

PCR was performed with 30 cycles (2 min at 94°C; 45 sec at 94°C for denaturation; 45 sec at 58°C for annealing; 1 min at 72°C for elongation; 10 min at 72°C). For each amplification, negative controls without template cDNA were run.

For separation of cDNAs, 1.5% agarose gels were used. 4 µl sample buffer was added. The samples were separated at 80 V for 60 min and visualized by UV fluorescence.

7.8 Quantitative real-time PCR for mRNA determination

Quantitative RT-PCR (or real-time RT-PCR) was performed with the LightCycler instrument. 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 5-fold 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, self priming formation, and primer melting temperature using the LightCycler Probe Design Software (Roche Diagnostics, Mannheim, Germany). 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 internal control. Based on these criteria, the following primers were used (i) 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; (ii) 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; (iii) 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 10-fold 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.

Authenticity of the amplified products was checked by dissociation analysis and agarose gel electrophoresis.

The individual level of initial target mRNA was expressed as the difference in C_T (i.e. the calculated fractional cycle number at which the fluorescence rises appreciably above background fluorescence) between control and sample (ΔC_T). The relative amount of target mRNA normalized to a reference gene (housekeeping gene) was calculated according to the following formula (Pfaffl *et al.*, 2002):

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

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

7.9 Analysis of intracellular amino acid concentrations

After washing and scraping of the cells in 0.5 ml buffer pH 7.4 per well of a 6-well-plate, three wells were unified to one sample and centrifuged (2000 rpm, 5 min, 4 °C). The supernatant was discarded and the pellet resuspended in 100 µl 10% (w/v) sulfosalicylic acid and incubated on ice for 30 min. After centrifugation (12200 rpm; 90 min; 4°C), the amino acid content of the supernatant was determined using the AA 45/32 Kit (Applied Biosystems, Toronto, Canada).

7.10 Analysis of intracellular ATP concentrations

ATP levels in OK cells were determined using an ApoSENSOR^{TM} Cell Viability Assay Kit (Biovision, Mountain View, California, USA). Cells were trypsinized, cell numbers were determined and 1000 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 and by modifying the concentrations for the standard curves: 0 mg/ml, 0.0001 mg/ml, 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, 1 mg/ml were used. Luminiscence was measured using a multiwell-plate reader (Luminoskan Ascent, Labsystems).

Cells were counted by using a hemocytometer. After trypsinization 20 µl of the cells were diluted with 80 µl Trypan Blue, the four big squares were counted and the cell number was calculated as follows: cell number/ml = (cells/small square) * 10^4 *5

7.11 Methods for determination of selenium

7.11.1 LC-MS/MS detection and quantification

7.11.1.1 Sample preparation

Cells were grown on 6-well-plates and cultured as described above. One to two days (OK cells) or 11 days (Caco-2 cells) after reaching confluency, cells were washed once with uptake buffer (UB, containing 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), incubated for 10 min with selenoamino acids (dissolved in UB), washed twice, scraped off in 0.5 ml double-distilled water/well and three wells were unified to one sample. After centrifugation (2000 rpm, 5 min, 4 °C), the supernatant was collected and measured, 50 µl of 6% sulfosalicylic acid was added to the pellets and incubated for 30 min on ice, the supernatant was centrifuged (13000 rpm, 5 min, room temperature). After centrifugation, the supernatants were neutralized with 25 µl 0.3 mM LiOH and measured in dilutions from 1:20 to 1:50.

7.11.1.2 Instrumentation and quantification

The LC/ESI-MS analyses for the quantification of organic selenium compounds were performed on a Agilent 1200 HPLC system (Waldbronn, Germany) consisting of a binary pump, an autosampler HTC PAL (CTC Analytics AG, Zwingen, Switzerland) coupled to a 3200 QTRAP with ESI (Applied Biosystems, Darmstadt, Germany). The analytical separation was performed on a HILIC column SeQuant (Haltern, Germany), (bead size, 5 µm; pore size 200 Å; 150 mm x 4.6 mm (i.d.)) using the mixture of 5 mM ammonium formate in 0.1% aequeous solution of formic acid and acetonitrile at a flow rate of 0.45 ml/min as gradient. Data acquisition was performed in a multiple reaction monitoring (MRM) mode: curtain gas of 15.0 psi, collision gas of 2.4 * 10^{-5} Torr vacuum gauge, ionspray voltage of 5500 V, temperature of 400 °C, ion source gas 1 and ion source gas 2 of 40.0 psi in positive ionization mode to generate $[M + H]^+$ ions. MSC was measured in negative ionization mode to generate $[M - H]^-$ ions by using following parameters: curtain gas of 15.0 psi, collision gas of 3.4 * 10^{-5} Torr vacuum gauge, ionspray voltage of -4500 V, temperature of 400 °C, ion source gas 1 of 35.0 psi and ion source gas 2 of 40.0 psi. The ions of three sets were monitored for the screening and quantifying of organic selenium compounds in the samples. Characteristic fragmentation of the selenoamino acids was observed and the ions generated as shown in Table 3 were used in MRM mode.

For quantification, external standards and the standard addition method were used to exclude matrix effects. For methods development, pure substances and for measurement two and three fragments, respectively, were used to unequivocally identify the substance. Taken

together, matches were made with the retention time, precursor ions, and the fragmentation patterns to identify the substance. Since the same fragments when measuring the samples in the current study were observed, this enabled identification of the compounds used.

The volumes of the pellets and the supernatants were determined and finally, the total content found in pellets plus supernatant was calculated and set in relation to cellular protein content.

Compound	Precursor ion *m/z*	Fragment ions *m/z*
MSC	181.8	94.6, 79.7
Se-Met	198.0	181.0, 56.1, 108.9
Se-Bet	169.0	109.9, 94.8
Se-Cys	335.0	246.0, 87.9

Table 3 - Characteristic fragments for selenoamino acids and ions used in MRM mode for quantification

7.11.2 Selenium determination and speciation via atomic absorption spectrometry (AAS)

Cells, cultured in 75 cm² flasks, were washed once with 4 ml of buffer pH 7.4 and uptake was initiated by adding 3 ml buffer pH 7.4 containing selenoamino acids. They were incubated for 10 min and uptake was terminated by the removal of the selenoamino acids followed by three times washing with ice-cold buffer pH 7.4. The cells were collected by scraping and resuspended in 2 ml uptake buffer. A 1-ml aliquot of cell sample was digested in a microwave 3000 (PerkinElmer, Ueberlingen, Germany) by 5 ml of nitric acid (65%) and 1 ml of hydrogen peroxide (30%). After cooling down, the extraction solution was transferred to a 25-ml-volumetric flask, filled up with double-distilled water and filtrated in the end. Se(VI) was reduced to Se(IV) after acid digestion: 10 ml of the solution were added to 5 ml hydrochloric acid (32%) and heated at 80°C for 20 min to convert Se(VI) to Se(IV). After cooling down, the solution was filled up to 50 ml with deionized water. Total selenium concentration of the pre-reduced solution was determined by an atomic absorption spectrometer with an electrically heated cuvette coupled to a flow-injection-hydride-system. Hydrochloric acid (3%) and a sodium borohydride solution (0.2% of sodium borohydride (w/v) and 0.5% of sodium hydroxide) were used to generate the selenium hydride. The resulting gas-/liquid-mixture was transferred and separated by an argon-flowed separator. With argon the metal-hydrides are transferred to the quartz cuvette for atomisation reaction and absorption measurements (Table 4).

Wavelength	196.0 nm
Gap length	2.0 mm
Measurement mode	atomic absorption
Signal conversion	peak height with background correction
Heated quartz cell temperature	900 °C
Smoothing	0.5 s
Integration time	15 s

Table 4 - Instrumental conditions for selenium determination

7.12 Calculations and statistical analysis

The apparent K_m values were calculated by non-linear regression of the Michaelis-Menten plot. For the increasing substrate concentrations, the concentration of the radiolabelled tracer remained constant, but counts were multiplied by the ratio of total substrate to radiolabelled substrate.

Calculations were done using the software Prism 4.0 (GraphPad Software, Los Angeles, CA, USA). Results were analyzed using unpaired Student's t-test and were considered statistically significant at a P value < 0.05. Data are given as means (± S.E.M.).

8 Literature

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9 Appendix

9.1 List of abbreviations

Å	ångstroem
AAS	atomic absorption spectrometry
act.	actinomycin D
AICAr	5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
α-ABA	α-aminobutyric acid
AMCA	7-amino-4-methyl-3-coumarinylacetic acid
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
Ci	Curie
cycloh.	cycloheximide
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
Fig.	Figure
g	gram
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK-3	glycogen synthase kinase 3
h	hour
^3H	tritium
HCl	hydrochloric acid
HEK	human embryonic kidney
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid
I_{max}	maximal currents
JNK	c-Jun N-terminal kinase
K_m	Michaelis constant
LC-MS/MS (tandem MS)	liquid chromatography-mass spectrometry

$LLCPK_1$	lewis lung carcinoma porcine kidney cells
MDCK	Maddin-Darby canine kidney
MeAIB	methylaminoisobutyric acid
MES	2-N-morpholinoethanesulfonic acid
µg	microgram
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
mm	millimeter
MOPS	3-(N-morpholino)-propansulfonic acid
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
nA	nanoampere
NaOH	sodium hydroxide
NBL-1 (MDBK)	Maddin-Darby bovine kidney
nM	nanomolar
OK	opossum kidney
PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
psi	pounds per square inch
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
S.E.M	standard error of the mean
SLC	SoLute Carrier
TAE	Tris-acetate-EDTA-buffer
TRIS	Tris-hydroxymethyl-aminomethane
V	voltage
V_{max}	maximal velocity

Abbreviations of amino acids are indicated as three letter codes.

9.2 Curriculum vitae

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Original papers and submitted manuscripts

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Nickel A., Klein U., Weitz D., Daniel H. (2009) L-proline uptake in renal OK epithelial cells – a second amino acid transport system is induced by amino acid deprivation. *Amino Acids* **in press**

Poster presentations

Nickel A, Kolmeder D, Weitz D, Daniel H. Overexpression of the proton-dependent peptide transporter PEPT2 in renal proximal tubule cells. The second EUGINDAT meeting, March 2005, Freising and 5th Transport Colloquium, May 2005, Rauischholzhausen.

Nickel A, Tudor S, Weitz D, Daniel H. Characteristics of luminal L-proline transport in renal proximal tubule cells. The second EUGINDAT meeting, March 2005, Freising and the Joint Meeting of The German Society of Physiology, March 2006, Munich.

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