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# Substrate-induced regulation of the intestinal peptide transporter

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*Life is what happens to you*
*while you are busy*
*making other plans*
*(John Lennon)*

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## Summary

The intestinal peptide transporter Pept1 plays an important role in the absorption of peptides and peptidometics from the small intestine. Whereas the transport properties of Pept1 have been studied in detail, little is known about the cellular mechanisms involved in the regulation of its activity. The aim of the present work was to investigate whether a prolonged exposure of *Xenopus* oocytes overexpressing r(abbit) Pept1 to dipeptide substrates affects the transport capacity of the protein. Electrophysiological measurements were used to determine transport currents and membrane surfaces (determined as electrical capacitance) of *X. oocytes* overexpressing rPept1, and these measurements were combined with immunofluorescence techniques.

The experiments presented evidence that insertion of peptide transporters into the membrane surface of oocytes resulted in an increase in membrane surface area paralleled by a rise in dipeptide-induced transport currents with a slope of about 11 nF/100 nA transport current. Prolonged exposure (4 hours / 3 days) of *X. oocytes* to various low concentrations or shorter exposure to high concentrations of the dipeptide Gly-Gln decreased transport activity markedly paralleled by a reduction in membrane capacitance. Immunofluorescent labelling of rPept1 confirmed that the reduction in surface area was due to a withdrawal of peptide transporter proteins from the oocyte membrane. A similar decrease in transport current and membrane capacitance was observed, when rPept1-expressing oocytes were exposed to the beta-lactam antibiotic cefadroxil or after activation of protein kinase C (PKC). The selective PKC-inhibitor bisindolylmaleimide I blocked the PKC-stimulated endocytosis but failed to inhibit the substrate-induced downregulation of rPept1 activity. The driving forces for maintaining absorptive dipeptide uptake mediated by Pept1 are the membrane potential, the substrate and the proton gradient. In search for the mechanism of the observed transporter downregulation a possible influence of the first two parameters in this process could be excluded. Intracellular pH ($pH_i$) measurements of rPept1-expressing oocytes indicated that after a incubation period of 4 hours in Gly-Gln $pH_i$ was lowered about 0.4 units. Exposure of oocytes coexpressing rPepT1 and the proton-dependent amino acid transporter PAT1 from murine (mPAT1) to either dipeptides or amino acids resulted in a decline in transport activity of both transporters supporting the hypothesis that long lasting changes in $pH_i$ play a triggering role in the endocytosis of peptide transporters. The results in this work suggest that the cotransported protons are accumulated intracellularly which may exceed the buffer capacity and acidify the cytosol, followed by the activation of yet unknown mechanisms that trigger the endocytosis of the transporter proteins.

# 1 INTRODUCTION

## 1.1 Mechanisms of epithelial nutrient transport

Epithelial cells form a barrier between the external and internal environment of an organism. Transport of solutes and water through this barrier occurs via passive and active transport systems.

Passive transport does not need chemical energy other than the chemical energy of the substrate itself. There are four kinds of passive transport: diffusion, facilitated diffusion, filtration and osmosis.

Diffusion is the movement of substances “down a concentration gradient” from an area of higher concentration to an area of lower concentration. Facilitated diffusion is permeation of larger, more hydrophilic molecules across a cell membrane by the help of special membrane carrier proteins. The speed of this process is limited by the number of carrier proteins. Filtration is movement of substances and water by means of hydrostatic pressure across pores of the cell membrane that may be considerably larger than the substance molecule itself. Osmosis is diffusion of water molecules to a region of higher solute concentration through the cell membrane impermeable for the solute, but permeable for water.

In contrast to passive transport, active transport requires external, additional e.g. chemical energy in form of ATP, allowing the transport of substances via specialised transport proteins against an electrochemical gradient. A distinction is drawn between primary, secondary and tertiary transport. In the simplest case, the transporter protein has itself ATPase activity. An example is the $Na^+/K^+$-ATPase, which transports 2 $K^+$ into and 3 $Na^+$ out of the cell, thus generating a $K^+$- and $Na^+$-gradient across the cell membrane. Due to the $K^+$ conductance present in most cell membranes, the unequal distribution of $K^+$ generates in addition an electrical gradient inside of the cell being negative as defined by the Nernst equation. Thus, both the chemical and electrical gradient of $Na^+$ is inwardly directed. This electrochemical gradient is used by further transport systems, called “secondary active” that transport substances “uphill” by coupling them to the “downhill” transport of $Na^+$. Examples of such systems are the sodium-dependent glucose transporter (SGLT) or the $Na^+/H^+$ exchanger (NHE). While SGLT transports glucose into the cells, NHE is carrying sodium ions into and protons out of the cell, thus building up a transmembrane proton gradient. Such a proton gradient is required for “tertiary active” transporters such as the proton-coupled amino-acid transporter (PAT) and the intestinal peptide transporter PEPT1 investigated in this thesis.

## 1.2 Overview of transport /exchange systems that serve the absorption of nutrients from the intestine

In the following sections, exchange and transport systems for the uptake of sugars, amino acids and peptides that are important for the present thesis are described. Fig. 1.2 represents a simplified scheme of nutrient transport and exchange systems in epithelial cells of the small intestine, including selected transport systems of the apical, but not showing the nutrient transport systems of the basolateral membrane.

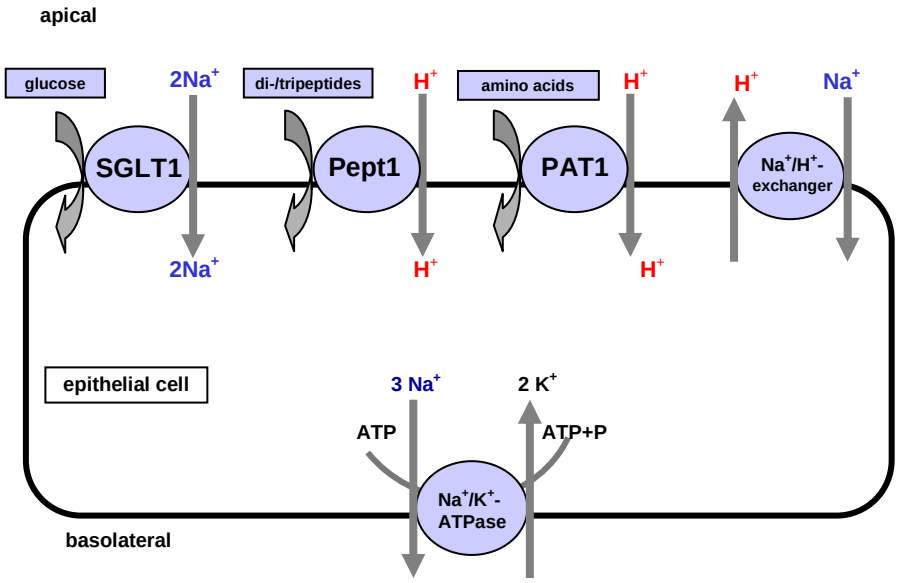


**Fig. 1.2:** Simplified scheme of nutrient transport and exchange systems in the apical membrane of epithelial cells

### 1.2.1 The sodium-dependent glucose transporter

Carbohydrates from the diet are digested to monosaccharides such as glucose, galactose and fructose. While fructose and, according to newer data, a considerable part of glucose are absorbed passively via the glucose uniporter GLUT5, uptake of glucose and galactose across the brush border membrane is facilitated by the $Na^+$-cotransporter family SGLT. Best characterised member of this family is the sodium-dependent glucose transporter termed SGLT1, first cloned in 1987 from rabbit small intestine (Hediger et al., 1987). SGLT1, a high affinity low capacity transporter, is located in the apical membranes of enterocytes and facilitates the active transport of sugars. Uphill transport, generating high intracellular concentrations of glucose, is driven by cotransport of two $Na^+$ ions into the

epithelial cell. $H^+$ and $Li^+$ can replace $Na^+$ as a driving ion, but with those cations the affinity for glucose is much lower. SGLT1 consists of 14 transmembrane domains (TMD's) with the N and C termini located extracellularly (Hirayama et al., 1994; Turk et al., 1994, Wright et al., 1994; Panayotova-Heiermann et al., 1997; Wright et al., 2003). SGLT2 another member of the $Na^+$-cotransporter family is located in the early proximal tubule segment S1. In contrast to SGLT1, SGLT2 is a low affinity high capacity transporter with a $Na^+$ to glucose ratio of 1:1. The human isoforms 1 and 2 show an identity of 95 % (Kanai et al., 1994; Wright, 2001).

### 1.2.2 $Na^+/H^+$ exchangers

$Na^+/H^+$ exchangers play an important role in intracellular pH homeostasis and cell volume regulation by carrying protons out and sodium ions into the cell in an electroneutral mode with a 1:1 stoichiometry. Furthermore, they build up a transmembrane pH gradient necessary to drive proton-coupled tertiary active transporters like Pept1 and PAT1. Seven isoforms termed NHE1 to NHE7 have been identified and cloned until yet. NHE1 is ubiquitously expressed, above all at the basolateral membrane of mammalian epithelial cells. Isoforms 2-4 are located at the apical membrane of epithelial cells, mainly in the intestine and in the kidney, whereas isoforms 5-7 are predominately expressed in the brain (Sardet et al., 1989; Orlowski et al., 1992; Tse et al., 1992; Masereel et al., 2003; Klanke et al., 1995). Most important for nutrient uptake across the brush border membrane is NHE3, since it generates, as already mentioned, the proton-gradient required for tertiary active transport systems.

Recently, an endogenous $Na^+/H^+$ antiporter in stage VI *Xenopus* oocytes was identified and cloned (Towle et al., 1991; Burkhardt et al., 1992; Busch et al.; 1998). However, the contribution of this isoform to the intracellular pH homeostasis in *Xenopus* oocytes is unclear until now. Towle et al. (1991) assumed that expression of this $Na^+/H^+$ antiporter might be negative affected by collagenase treatment of oocytes and length of time after removal of oocytes.

### 1.2.3 Amino acid transporters

A broad scope of amino acid transporters contribute to the uptake of differently charged amino acids in intestinal epithelia. The main $Na^+$-dependent amino acid transporters at the apical membrane are the system $B^0$ for neutral amino acids, system $B^{0,+}$ for neutral and basic amino acids, system asc for alanine, proline and glycine (Munck et al., 2000). Anionic amino acids are absorbed by the $Na^+$- and $K^+$-dependent system $X^-_{AG}$ (Munck et al., 2000) The $Na^+$-independent system $b^{0,+}$ serves the uptake of neutral and dibasic amino acids in exchange for intracellular neutral amino acids (Munck et al., 2000; Palacin et al., 1998). In the following, focus is put on the amino acid transporter PAT1, a member of the amino acid/auxin permease family. In contrast to many other amino acid transporters, the driving force for uphill transport in this system is not the $Na^+$- gradient, but is provided by $H^+$-ions. Protons and substrates are cotransported with a stoichiometry of 1:1 (Boll et al., 2002, Boll et al., 2003 a). Substrates that are accepted and transported by PAT1 are small L-α-amino acids, D-amino acids, β-alanine, γ-aminobutyric acid (GABA), betaine and selected short-chain fatty acids (Boll et al., 2003 b; Chen Z et al., 2003; Foltz et al., 2004). By now, four members of this family termed PAT 1 – 4 have been cloned and partly characterised, but only PAT1 is expressed at the apical membrane of the small intestine. All members of this transporter family consist of 483 – 500 amino acids and 11 TMD (Boll et al., 2002). Until now, PAT1 has been cloned and characterised from rat, mouse and human (Sagne et al, 2001; Boll et al., 2002; Boll et al., 2003 a; Chen Z et al., 2003).

At the basolateral membrane, dibasic amino acids leave the cell via $Na^+$-dependent type $y^+L$ transporters whereas neutral amino acids are released by the $Na^+$-independent systems asc and L. Intracellular glutamate is exchanged against cystine via the systems $x^-_c$ and asc (Wagner et al. 2001, Verrey et al., 2004).

### 1.2.4 Peptide transporters

#### 1.2.4.1 Historic aspects and cloning of peptide transporters

For long time, it has been believed that dipeptides are completely hydrolysed in the gut lumen and are absorbed exclusively as free amino acids. In the early seventies, however, perfusion studies of the small intestine with dipeptide-containing solutions showed evidence for absorption of intact dipeptides (Adibi et al., 1968; Adibi, 1971). In vitro studies demonstrated that hydrolytic enzymes are predominantly concentrated in the

cytosol whereas only a few enzymes against dipeptides are found extracellularly in the brush border region (Adibi, 1971; Peters et al. 1970). In 1974, the existence of a carrier system for dipeptides in mammalian intestinal cells completely different from the carrier system for free amino acids has been demonstrated (Adibi et. al., 1974). Furthermore, it has been shown that the uptake of di- and tripeptides across the brush-border membrane is an active and selective process and that not only dipeptides but also tripeptides are absorbed in intact form (Addison et al. 1972; Addison et al., 1975; Adibi et al., 1974).
Later, evidence has been provided that active uptake of di- and tripeptides via selective carriers is not restricted to the intestine but also occurs in the kidney (Adibi, 1977, Ganapathy et al., 1981; Ganapathy et al., 1986).

In the early eighties, studies on brush border membrane vesicles (BBMV) indicated that the renal and intestinal peptide transport is independent of $Na^+$, $Cl^-$ or $K^+$ ions but electrogenic and coupled to an inward current of protons (Berteloot et al., 1981; Ganapathy et al., 1981, Ganapathy et al., 1985; Ganapathy et al., 1984).

In 1994, a break through was achieved by expression cloning of the first mammalian peptide transporter from human and rabbit small intestine termed Pept1 (Boll et al., 1994; Fei et al., 1994). During the following years, peptide transporters from rat (Saito et al., 1995), mouse (Fei et al. 2000) sheep (Pan et al., 2001) and zebra fish (Verri et al., 2003) have been cloned. The renal isoforms of $H^+$/peptide transporters termed Pept2 has been identified and characterised from human (Liu et al., 1995), rat (Saito et al., 1996), rabbit (Boll et al., 1996) and mouse (Rubio-Aliaga et al., 2000). Pept2 is a “high affinity – low capacity” transporter whereas Pept1 exhibits low substrate affinities but high capacities (Silbernagl et al., 1987).
Until the beginning of 90’s, it has not been known, whether the absorbed peptides enter the portal blood in intact form or after hydrolysis by peptidases, as free amino acids via basolateral-located peptide or amino acid transport systems. Meanwhile, evidence has accumulated that at least a part of the absorbed peptides leave the cell on the basolateral side without being hydrolysed in the cell (Terada et al., 1999; Sawada et al., 2001). Controversial is however, whether the basolateral peptide transporter is a facilitative or an $H^+$-coupled transport system (Dyer et al., 1990; Inui et al., 1992 a; Saito et al., 1993; Thwaites et al., 1993 a; Thwaites et al., 1993 b; Matsumoto et. al., 1994; Terada et al., 1999).

1.2.4.2 Tissue distribution of peptide transporters

In situ hybridisation studies revealed that Pept1 is expressed in the intestinal epithelium of rabbit. Immunohistochemical observations in rat tissue indicated localisation of Pept1 in the digestive tract, above all in the duodenum, jejunum and ileum. High levels of Pept1 mRNA were found in duodenal and jejunal enterocytes with lower levels in the ileal epithelium. Expression of Pept1 was detected in the brush border membrane of epithelial cells where it was most abundant in the villi (Freeman et al., 1995; Ogihara et al., 1996). Previous studies provided evidence that Pept1 may also be located in extraintestinal tissues. Immunolocalisation experiments found evidence for Pept1 expression in S1 segments of the proximal tubule (Shen et al., 1999). *Bockmann et al.* (1997) reported localisation of Pept1 in nuclei of vascular smooth cells and Schwann cells as well as in lysosomes of the exocrine pancreas. Immunostaining of cells of widely used cell lines, like Caco2 cells, HEK293 cells and HepG2 cells indicated expression of Pept1 at the lysosomal membrane of these cells (Lee at al., 1999). The authors assumed that Pept1 plays a role in the lysosome-to-cytoplasm transport of di- and tripeptides, resulting from protein catabolism.

The high-affinity isoform Pept2 shows a much broader tissue distribution within the organism. It was found in kidney tubule segments S2 and S3 (Boll et al., 1996; Shen et al., 1999), in the colon, spleen and pancreas as well as in the peripheral and central nervous system (Döring et al., 1998 b; Groneberg et al., 2001 a; Berger et al., 1999). In the lung, Pept2 is expressed in type II pneumocytes and in the apical membrane of tracheal and bronchial epithelial cells (Groneberg et al., 2001 b). Localisation of Pept2 was also detected in epithelial cells of the ducts and glands in the lactating mammary gland of rats (Groneberg et al., 2002).

1.2.4.3 Substrate specificity of peptide transporters

Peptide transporters catalyse the active transport of a broad spectrum of di- and tripeptides. Free amino acids and larger peptides are not recognised. By using ω–amino fatty acids (ω–AFA) as model compounds, *Döring et al.* (1998 a) determined the minimal structural requirements for substrates to be recognised by Pept1. Computational analysis combined with uptake assays in transgenic yeast cells and *Xenopus laevis* oocytes expressing Pept1 demonstrated that the presence of at least one amino- and one carboxyl group is a minimal prerequisite for substrates to be accepted and transported by Pept1, whereas a peptide bond has not necessarily to be a part of the molecule. The two ionised

headgroups have to be separated by four or more methylene groups with a distance of > 500 pm and < 630 pm. Removal of either the amino group or the carboxyl group in ω–AFA abolished electrogenic transport features. In addition to peptides a wide range of peptide-like drugs meet these requirements such as β-lactam antibiotics (aminopenicillins, aminocephalosporins), angiotensin-converting enzyme-inhibitors (enalapril), renin inhibitors (S86, 3390) and the peptidase inhibitor bestatin (Daniel & Adibi, 1993; Wenzel et al. 1995; Dantzig et al., 1997; Bretschneider et al., 1999; Lin et al., 1999; Zhu et al., 2000; Kramer et al., 1990; Inui et al., 1992 b; Daniel & Adibi, 1994). It also could have been shown that amino acid aryl amides are recognized and even transported by Pept1 (Daniel & Adibi, 1994; Börner et al., 1998). Delta-aminolevulinic acid (ALA), a precursor of porphyrin synthesis used as an endogenous photosensitizer for photodynamic therapy, was reported to be transported by Pept1 and Pept2 expressed in *Xenopus laevis* oocytes or *Pichia pastoris yeast* in an electrogenic mode (Döring et al., 1998 b).

Profound insight into the structural requirements of peptide transport enabled the design of pharmacologically active peptidometics that are accepted by Pept1 such as L-Dopa-L-Phe, a therapeutic agent for Parkinson disease and L-valacyclovir, an antiviral active prodrug of acyclovir (Tamai et al., 1998; Lee at al., 1996; Ganapathy et al., 1998).

The substrate spectrum transported by Pept2 is basically similar to that of Pept1. However, Pept2 is a “high affinity – low capacity” transporter whereas Pept1 exhibits low substrate affinities but high capacities (Silbernagl et al., 1987). Studies in renal BBMV elucidated the relationship between structural features and affinity of substrates for interaction with Pept2. To maintain high affinities, the existence of both a free amino and carboxyl terminus in the substrate is necessary. Besides, the terminal amino group and the peptide bond nitrogen have to be located in the α-position. Trans-configuration rather than cis-configuration is preferred and the affinity of oligopeptides increases with more hydrophobic side chains (Daniel et al., 1992). It also could have been shown that the aminopeptidase inhibitors arphamenine A and B, in which the peptide bond is replaced by a ketomethylene function, interact with the substrate binding site of the renal peptide transporter without diminishing the affinity (Daniel & Adibi, 1994).
Generally, in addition of these head groups, size, charge and orientation of side chains, L-isomers of oligopeptides display a higher affinity for peptide transporters than the D-enantiomers (Daniel et al., 1992; Lister et al., 1995; Wenzel et al., 1995; Meredith et al., 2000).

1.2.4.4 Molecular structure of peptide transporters

Peptide transporters belong to the proton-coupled oligopeptide transporter family SLC15A. Members of this family are Pept1, Pept2 and the peptide/histidine transporters PHT1 and PHT2. All of these four transporter proteins consist of 12 membrane-spanning domains with a large hydrophilic extracellular loop between transmembrane helices 9 and 10 (Covitz et al. 1998, Yamashita 1997) (Fig. 1.2.4.4). Conserved regions are primarily found within the first 6 transmembrane domains whereas N and C termini, both are located intracellular, exhibit the largest differences in amino acid sequence (Covitz et al., 1998, Fei et al 1998; Daniel, 1996). The human intestinal peptide transporter isoform 1 (hPept1) is encoded by a gene consisting of 24 exons and is located on chromosome 13. It consists of 708 amino acids and a core molecular mass of ~79 kDa. hPept1 has 7 N-glycosylation and 2 protein kinase C (PKC) consensus sites but is missing protein kinase A (PKA) or tyrosine kinase (TK) consensus sites (Liang et al, 1995).
The gene encoding for human Pept2 is situated on chromosome 3 and has a molecular mass of ~82 kDa. It consists of 729 amino acids, 3 N-glycosylation sites, 7 protein kinase C (PKC) sites and, in contrast to Pept1, has one protein kinase A (PKA) and one tyrosine kinase (TK) phosphorylation site. The amino acid sequences of both transporters are about 50 % identical (Ramamoorthy et al., 1995; Liu et al., 1995; Daniel, 1996).

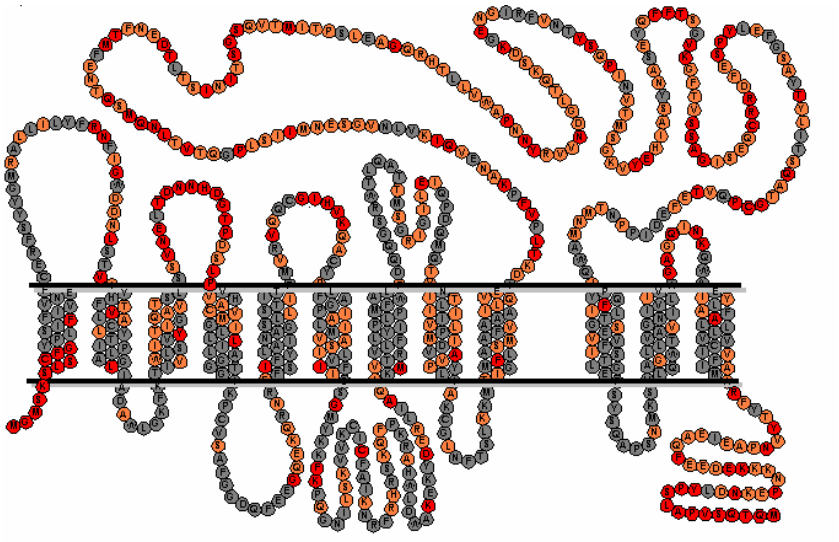

**Fig. 1.2.4.4:** Proposed structure of the intestinal peptide transporter Pept1

Approaches to identify the substrate-binding site of peptide carriers were performed by creating Pept1/Pept2 chimeras. Functional regions that are responsible for substrate recognition, substrate affinity and pH-dependency of Pept1 and Pept2 seem to be located

in the N-terminal region of the transporter proteins. In contrast, the C-terminal region might be important for protein trafficking or regulation of transport activity of peptide transporters (Terada et al., 2000; Döring et al., 1996; Döring et al., 2002).

In addition to this “large-scale” description single amino acids such as histidine have been identified by treatment with diethylpyrocarbonate (DEPC), a histidine modifier, or by site-directed mutagenesis to be essential for the catalytic function of peptide transporters. Treatment of brush-border membrane vesicles from renal cortex or from renal epithelial cells (LLC-$PK_1$) with DEPC completely abolished dipeptide uptake. Modification by DEPC was inhibited by simultaneous application of substrates consisting of an α-amino group, showing that histidyl residues are no more accessible for DEPC. Peptide-like drugs without an alpha-amino group such as bestatin or ceftibuten failed to prevent the DEPC-induced inhibition. The finding suggests that the alpha-amino groups of dipeptides or peptide-like drugs interact with histidine residues of Pept1 or Pept2 and may be involved in substrate recognition and binding by the peptide transporters (Miyamoto et al., 1986; Kramer et al., 1988; Terada et al., 1996, Terada et al., 1998). In Pept1 three histidine residues (His-57, His-121 and His-260) are conserved. Since Pept2 has a longer amino acid sequence at the N-terminal, the corresponding residues in Pept2 are His-87, His-142 and His-278. Site-directed mutagenesis of His-57 in Pept1, respectively His-87 in Pept2 completely destroyed transport activity, identifying these residues as proton-binding sites of Pept1 and Pept2. His-260 is not considered to be important in binding and translocation of protons, whereas the role of His-121 in Pept1 is controversial. *Chen et al. (2000*) proposed that His-121 is involved in the binding of protons and substrate recognition whereas a study of *Fei et al.* (1997) indicated that mutation of His-121 did not affect transport function of Pept1. His-142 in Pept2 is not essential for transport function since the mutated protein still exhibits transport activity (Terada et al., 1996; Fei et al., 1997; Chen et al., 2000; Uchiyama et al., 2003).

In addition to histidines, other residues were also identified as important. Tyrosine 167 in transmembrane domain (TMD) 5 seems to be important for the function of Pept1. Site-directed mutagenesis of tyrosine 167 to alanine completely abolished Gly-Sar uptake in HEK293 cells transfected with Y167A-hPept1 (Bolger et al., 1998, Yeung et al 1998). However, western blot analysis and immunofluorescence microscopy did not detect any differences in membrane expression levels of Y167A-hPept1 compared to the wild type. Thus, mutation of Y167 did only diminish transport activity but neither expression level nor trafficking of Pept1 to the plasma membrane (Yeung et al., 1998) were affected.

Another residue of interest is arginine at position 282, a highly conserved charged residue in transmembrane 7 of Pept1 sequence. Mutation of arginine to glutamate reduced uptake activity by about 43% suggesting that the positive charge is important at amino acid position 282 (Kulkarni et al., 2003). *Meredith D*. (2004) predicts that R282E uncouples the cotransport of protons and peptides and generates a peptide-controlled cation channel.

#### 1.2.4.5 Substrate/proton stoichiometry of peptide transporters

The substrate/proton stoichiometry was determined by comparing the uptake of radiolabelled dipeptides with the dipeptide-induced inward current of the same oocyte under voltage clamp conditions. The measured stoichiometry of Pept1 for neutral and cationic substrates is 1:1 (Daniel et al., 1991; Mackenzie et al., 1996; Steel at al., 1997; Kottra et al., 2002; Irie et al., 2004). Concerning the stoichiometry of anionic substances, however, different hypothesis, exist. Since also the transport of anionic substrates generates positive inward currents, the question is whether an anionic substrate is protonated before being transported or whether an $H^+$-ion binds to the transporter molecule during transport of the anionic substrate. *Mackenzie et al.* (1996 b) suggested an exchange mechanism, i.e. that one negatively charged ion and the anionic substrate with one $H^+$-ion are transported simultaneously and in opposite directions. A study by *Steel at al.* (1997) suggests that before transport, an anionic substrate is protonated, resulting in a substrate-to-proton ratio of 1:2. Recently, a computational model by *Irie et al.* (2005) predicted that $H^+$ binds not only to the proton-binding site but also to the substrate-binding site. Thus, binding of $H^+$ to both proton- and substrate-binding site inhibits the transport of neutral and positively charged substrates but is necessary for the uptake of negatively charged substrates, leading to a stoichiometry of anionic substrate to proton of 1:2 (Irie et al., 2005). Previous studies in our laboratory showed evidence that both the charge of the dipeptide as well as the position of the charged amino acid chain in the substrate affect the proton/substrate stoichiometry (Kottra et al. 2002). *Chen et al.* determined for Pept2 a proton to substrate stoichiometry of 2:1 for neutral and 3:1 for anionic dipeptides. For the cationic dipeptide Phe-Lys a stoichiometry of 1.4:1 was calculated, suggesting that the dipeptide may be transported either in neutral (deprotonated) form (60 %) or in positively charged form (40 %).

# 2 METHODS AND MATERIALS

## 2.1 The *Xenopus laevis* oocyte expression system

### 2.1.1 Handling of *Xenopus laevis*

Adult *Xenopus laevis* femals were purchased from *African Xenopus facility* (Krysna, South Africa) and kept in opaque tanks covered with a wire grid. The animals were fed with ox liver and special frog pellets once a week. Two days after feeding, the tanks were cleaned and refilled with fresh water to avoid bacterial contamination. Since *Xenopus laevis* are very sensitive to climate changes, room temperature were constantly kept to 20 – 22 °C with a 12 h light / 12 h dark cycle.

### 2.1.2 Isolation and preparation of *Xenopus laevis* oocytes

Animals were anesthetized with 0.8 g MS-222 (3-aminobenzoic acid ethyl ester) dissolved in cold water (1.5 l). The frog was leaved under anesthetic until it did not react anymore to a leg pinch test. During surgery the animal was kept on ice. After a small incision (1 cm) into the abdominal cavity several lobes of ovary were removed and directly placed into a petri dish with calcium-free ND96 solution. The isolated ovarian tissue was minced into small pieces and repeatedly washed with calcium-free ND96 solution. Thereafter, oocytes were defollicated with 1.72 mg/ml collagenase A by gentle agitation for 90 min at room temperature. After defollication oocytes were washed several times initially with calcium-free ND96 and then with standard ND96 solution to remove any remaining enzymatic activity. Remaining parts of the follicle layer of stage V and VI oocytes were removed mechanically with fine forceps, apoptotic and damaged oocytes were discarded. Healthy oocytes were maintained at 18°C in regular ND96 solution supplemented with 100 µg/ml gentamycin and 5 mM sodium pyruvate overnight.

The frog was allowed to recover from operation in a separate tank. After final surgery, frogs were killed by an anaesthetic overdose.

### 2.1.3 Microinjection of cRNA and maintainance of *Xenopus* oocytes

cRNA was injected one day after surgery with a Drummond nanoinjector. A fine glass capillary (tip diameter ≤ 20 µm) was backfilled with mineral oil and assembled onto the nanoinjector and loaded with cRNA in a concentration of 1 µg/µl. Healthy and undamaged oocytes were placed on a small grating and punctured with the microcapillary. Tab. 2.1.1 shows the injected amount of cRNA per oocyte.

| cRNA | injected amount (ng) |
|---|---|
| rPept1 | 18.2 |
| mPAT1 | 13.2 |
| hSGLT1 | 23.0 |

**Tab. 2.1.1:** Injected amounts of rPept1, mPAT 1 and hSGLT1 cRNA per oocyte

For coexpression experiments two different kinds of cRNA were injected into one single oocyte. For this purpose, a certain volume of each cRNA were mixed thoroughly and pulled up into the microcapillary for injection. Tab. 2.1.2 represents the amount of the coinjected cRNAs per oocyte.

| amount of cRNA / coinjected types of cRNA | rPept1 (ng) | mPAT1 (ng) | hSGLT1 (ng) | total amount of cRNA (ng) per oocyte |
|---|---|---|---|---|
| rPept1 + mPAT1 | 13.2 | 11.5 | - | 23 |
| rPept1 + hSGLT1 | 9 | - | 18 | 27 |
| mPAT1 + hSGLT1 | - | 11.5 | 11.5 | 23 |

**Tab. 2.1.2:** Amount of coinjected cRNAs per oocyte

After injection of cRNA, oocytes were maintained in ND96 solution supplemented with gentamycin and sodium butyrate at 18 °C. Medium was changed daily. Oocytes were used for experimental procedures 3 – 5 days after injection of cRNA.

## 2.2 Molecularbiological and biochemical methods

The cDNA encoding for the peptide transporter Pept1 from rabbit has been cloned in the pSport1 vector, which is under the control of a T7 promotor and confers ampicilline resistance for colony selection and growth. The 3' end of the rPept1 sequence contains a poly A tail to improve cRNA stability and protein yield.

### 2.2.1 Transformation of cDNA in competent *Escherichia coli* (*E. coli*) cells

100 µl of *Escherichia coli* strain Top 10 F' competent cells were mixed with 5 µl of the rPept1-pSport1 plasmid, incubated on ice for 30 min and after that heat pulsed at 42 °C for 90 s to increase the permeability of the bacterial cell membrane. The reaction sample was then placed on ice for 2 min. After addition of 1 ml of Luria Bertoni (LB) medium the reaction mix was incubated at 37 °C for 1 hour and then plated on selective LB-agarose dishes containing 0.1 mg / ml ampicillin. The next day, several clones were picked from the agarose dishes and grown at 37° C overnight und er agitation in 2 ml of LB medium supplemented with 0.1 mg / ml ampicillin.

### 2.2.2 Preparation of cDNA

Extraction of the plasmid cDNA was performed using the *flexi Prep*$^{TM}$ kit. An overnight culture of *E. coli* was transferred to a microcentrifuge tube and centrifuged for 30 s at 10000 rpm speed to pellet the cells. The supernatant was removed and the bacterial cells were resuspended in an isotonic solution. Cells were lysed by alkali treatment and chromosomal DNA and proteins were denatured. pH was neutralized using a potassium acetate solution. After centrifugation for 5 min at full speed the supernatant was transferred to a clean centrifuge tube. Plasmid DNA was concentrated by isopropanol precipitation and pelleted by a centrifugation step for 10 min at full speed. The supernatant was discarded and the pellet was washed with ethanolic washing buffer and 70 % ethanol to remove contaminants. Supernatant was removed and the pellet desiccated. DNA was eluted in 50 µl sterile water and DNA yield was measured photometrically.

### 2.2.3 Linearization of cDNA

Since in vitro transcription requires a linearized plasmid DNA, DNAs were cleaved with the respective restriction enzymes: rPept1 with *Not I*, hSGLT1 with *Xba I* and mPAT1 with *Hind III*. Therefore, 6 µg DNA, 4 µl 10 x reaction buffer and 2 µl restriction enzyme were filled up with sterile water to a volume of 40 µl and incubated for at least 3 - 4 hours at 37° C. The linearized DNA was purified by phenol extraction. A one-half volume of a 1:1 mixture of phenol/chloroform was added to the sample followed by vigorous mixing and centrifugation for a few minutes to separate the phases. The upper (aqueous) phase was carefully transferred to a new tube and extracted with ammonium acetate solution (5 M ammonium acetate, 100 mM EDTA) at -20°C overnight. The sample was then centrifuged at 12000 rpm at 4° C for 15 min, washed with ice-cold 70 % ethanol and again centrifuged. The supernatant was removed, the pellet air-dried and eluted in 16 – 20 µl sterile water.

### 2.2.4 Transcription of cRNA

For in vitro transcription of rPept1, hSGLT1 and mPAT1 cRNA the *mMessage mMachine* transcription kit was used. 10 µl 2 x ribonucleotid mix, 2 µl reaction buffer, 1 µg linearized cDNA, 2 µl enzyme mix were filled up to a volume of 20 µl with nuclease-free water and thoroughly pipetted up and down. The reaction mixture was then incubated at 37° C. After 2 hours, 1 µl DNAse was added to remove template DNA. After further 15 min, the reaction was stopped and the RNA precipitated by adding 30 µl of nuclease-free water and 30 µl LiCl solution (7.5 M lithium chloride, 50 mM EDTA). The reaction mixture was incubated at -20° C overnight. To pellet the RNA, the mixture was centrifuged for 15 min at 4° C. After removing the supernatant, the pellet was washed with 200 µl cold 70% ethanol and re-centrifuged for 15° C to remove unincorporated nucleotides completely. Ethanol was removed, pellet air dried and resuspended in 16 µl sterile water. cRNA concentration was determined with a photometer, diluted to a concentration of 1 µg / µl and then frozen at -80° C.

### 2.2.5 Insertion of a FLAG epitope into rPept1 sequence

Insertion of a FLAG eptitope (DYKDDDDK) into the large extracellular loop between the transmembrane domains 9 and 10 of rPept1 sequence enabled to determine the expression level of tagged peptide transporters at the membrane surface of the oocytes by immunofluorescence methods. This technique is a useful tool when combined with TEVC measurements to investigate whether changes in transport currents are caused by a change in the amount of expressed transporter proteins due to exo- or endocytotic processes. Selective detection of the tagged transporters at the oocyte surface was performed with a specific primary antibody against the inserted epitope and a secondary fluorescence-labelled antibody. Fluorescence intensity was detected by confocal microscopy.

#### 2.2.5.1 Insertion of a *bgl II* restriction site into rPept1 sequence by site-directed mutagenesis

To enable insertion of the FLAG epitope into the rPept1 sequence, first, a *bgl II* restriction site had to be introduced into the multiple cloning site by site-directed mutagenesis. This restriction site was inserted at position 543 in rPept1 (pSport1) sequence by changing glutamate to arginine.

Site-directed mutagenesis was perfomed with the *QuikChange*® *Site-Directed Mutagenesis* Kit using a DNA polymerase and a temperature cycler.
For polymerase chain reaction (PCR), the following oligonucleotides, encoding the *bgl II* restriction site, were performed.

forward primer:
5'GAG CAG TGC AGG CGG GAC TTT AGA TCT CCG TAC CTG GAG TTT GGC-3'

backward primer:
5'-GCC AAA CTC CAG GTA CGG AGA TCT AAA GTC CCG CCT GCA CTG CTC-3'

PCR was carried out using 5 µl 10 x reaction buffer, 10 ng dsDNA (double-stranded DNA), 125 ng of both forward and backward primer and 1 µl dNTP. Sterile water was added to a

final volume of 50 µl. Finally, 1 µl of *Pfu Turbo* DNA polymerase (2.5 U / µl) was pipetted to the reaction mix.

Temperature cycling was set as follows: The reaction mix was heated to 95° C for 30 s followed by a hybridization step at 55° C for 1 min and an elongation step at 68°C for 16 min. This cycle was repeated 12 times and finally, reaction mix was cooled down to 4° C.
After temperature cycling 1 µl of the restriction enzyme *dpn I* (10 U/µl) was added to the amplification reaction and incubated at 37° C for 1 hour to digest the supercoiled dsDNA.

#### 2.2.5.2 Treatment of DNA with alkaline phosphatase

Shrimp alkaline phosphatase (SAP) was used to remove 5'-phosphat groups from Plasmid-DNA thus avoid self-ligation of the vector ends. Therefore, 1 µl SAP was added to a 20 µl reaction mix and incubated for 1 hour at 37° C.

#### 2.2.5.3 Gene-clean of the modified *bgl II*-rPept1 cDNA

Extraction and purification of DNA from agarose gels were done with the *QIAquick Gel Extraction* kit. In principle, in the presence of high salt concentrations, DNA adsorbs to a silica-membrane whereas contaminants flow trough a column.
The DNA sample was loaded on a native agarose gel at 60 V. Separated DNA fragments were excised from the gel with a scalpel and transferred to a reaction tube. After determining the sample volume the threefold volume of binding buffer was added to the sample. The gel slice was incubated at 50° C until it was completely dissolved. To bind DNA, the sample was transferred to a column and centrifuged for 1 min at full speed. Traces of agarose and salts were washed away by adding of ethanol-containing washing buffers. After discarding the flow-through, the sample was centrifuged at 10 000 rpm for 1 min and DNA eluted in 50 µl sterile water.

2.2.5.4 Primer annealing

The complementary oligonucleotides encoding for the FLAG epitope were annealed to facilitate its insertion into the rPept1 sequence. Therefore, 1 µg of both forward and backward primer, 5 µl One-Phor-All buffer and sterile water were added to a reaction volume to 50 µl. The reaction mix was incubated for 10 min at 70° C and then cooled down to less than 35°C.

FLAG-tag

forward primer: 5'-GATCC GATTATAAAGATGATGATGATAAA G-3'
backward primer: 5'-GATCCT TTATCATCATCATCTTTATAATCG-3'

2.2.5.5 Ligation by use of T4 DNA ligase

The annealed oligonucleotides were ligated with a T4 DNA ligase into the *bgl II* restriction site of rPept1 sequence. First of all, the *bgl II*- modified rPept1 cDNA, was linearized with the restriction enzyme *bgl II* to allow the ligation of the primers, encoding for the FLAG epitope.

Ligation was performed, using 50 ng linearized rPept1 cDNA, 0.7 ng of annealed primers, 1 µl 10 x ligase buffer, 1 µl T4 DNA ligase, filled up to a volume of 20 µl with sterile water. The ligation mix was incubated for 2 hours at room temperature.

2.2.5.6 Transformation of the ligated plasmid into competent cells and preparation of cDNA

Transformation of the ligated plasmid into competent cells and preparation of cDNA was performed using the protocol described in chapter 2.3.1.

2.2.5.7 DNA sequencing

Sequencing of the rPept1-FLAG DNA was performed using the *chain termination* method by Frederick Sanger. DNA was detected by Cyanin5 dye-labelled didesoxynucleotides (ddNTPs). The *Thermo Sequenase Cy5 Dye Terminator* Kit was used for DNA cycle sequencing, providing a thermostable polymerase, Cy5-labelled ddNTPs and dNTPs and

a single unlabelled primer. Thermal cycles of denaturation and polymerization generated products that terminate with the Cy5-labelled ddNTPs. For each template four separate Cy5 dideoxy reactions were needed. The reactions were loaded on four adjacent lanes on an sequencing gel and were electrophoresed with a *ALFexpress DNA* sequencer. Data were analyzed with the *ALFwin Sequence* analyzer 2.1 software by comparing the DNA sequences with the Pept1 sequence availaible at the gene data base (www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nuccore&id=126723676).

#### 2.2.5.8 Functional testing of the FLAG-tagged rPept1 transporter proteins

To ensure that the FLAG-tagged rPept1 transporter proteins are inserted into the oocyte plasma membrane and are functional, transport parameters were determined by two-electrode voltage clamp (see below). Maximal transport currents ($I_{max}$) and the Michaelis-Menten affinity constants ($K_m$) were calculated, and indicated no differences in expression and transport parameters between wild-typ and FLAG-tagged rPept1 expressing oocytes.

#### 2.2.5.9 Fluorescence detection of FLAG-tagged rPept1 transporters expressed in *Xenopus* oocytes

All stages of this protocol were performed on ice (4° C) to prevent trafficking of peptide transporters to and from the plasma membrane.
3 days after injection of FLAG-tagged rPept1 (rPept1-FLAG) cRNA in *Xenopus* oocytes, 20 – 30 oocytes per group were transferred into 6-well plates and incubated in modified ND96 solution containing 1 % bovine serum albumin (BSA) for 30 min at 4° C under slight agitation. Thereafter, oocytes were incubated in 10 µg/µl of the monoclonal primary antibody Anti-FLAG M2 for 1 hour at 4℃ in ND96/BSA . Oocytes were washed 8 times with ND96/BSA to remove unincorporated primary antibodies followed by incubation in 10 µg/µl goat anti-mouse lgG secondary antibody labelled with Alexa Fluor 488. Oocytes were washed thoroughly 6 times in ND96/BSA and then 8 times in ND96 without BSA.
For fluorescence detection, oocytes were placed on glass cover slips and embedded in few drops of ND96 solution. Fluorescence intensity was measured with a Leica TCS SP2 confocal microscopy at an excitation wavelength of 488 nm and a detection wavelength of 500 – 535 nm. Total fluorescence intensity was calculated from 10 optical sections determined on each half of the oocyte.

## 2.3 Electrophysiological methods

### 2.3.1 Two electrode voltage clamp technique

Oocytes were placed in a small perfusion chamber with a volume of about 0.5 ml. The supply of different solutions was regulated by solenoid valves. During measurement, oocytes were continuously superfused with ND96 solution or substrate-containing solutions. The microelectrodes used for electrophysiological measurements were made from borosilicat glass capillaries. The capillary tip was pulled with a Zeitz electrode puller (München) and had a diameter between 10 – 15 µm with a resistance between 0.8 and 1.2 MΩ. After oocytes were impaled with a potential and a current electrode the spontaneous membrane potential was controlled and only those oocytes with a potential higher than -15 mV were used for electrophysiological measurements. Thereafter oocytes were voltage-clamped at a holding potential of -60 mV. Transport currents were measured with a TEC-05 amplifier and the CellWorks software. Current-voltage (IV) relations were determined in the range of -160 to +80 mV. The current generated by the substrate transport at a certain membrane potential was calculated as the difference of the transport currents evoked in the absence and presence of substrate. Transport currents were measured, when not otherwise noted, by application of 5 mM Gly-Gln. Since the $K_m$ value of this substrate is about 0.7 mM, the above mentioned concentration induced nearly maximal transport currents.

### 2.3.2 Membrane capacitance measurements

Membrane capacitances ($C_m$) were determined by a method adapted from Schmitt & Koepsell (2002). The capacitance of voltage clamped oocytes was calculated from current jumps elucidated by membrane potential ramps. The potential function was composed of two asymmetrical depolarizing and hyperpolarizing ramp pairs. Starting at the holding value of -60 mV the potential was changed to -40 mV (slope 2 mV/ms) and then returned to the holding value (slope -1 mV/ms). After a short delay a hyperpolarizing ramp-pair with a peak of -80 mV and otherwise identical parameters was initiated. To calculate the membrane capacitance, the theoretical height of the current jumps was calculated by fitting linear functions to the portions of the current signal sufficiently far away from the transition region and extrapolating these functions to the end point of the ramp. In

accordance with the time course of potential six current jumps were fitted by Excel macros and the mean value of the six calculated capacitances (usually not differing more than ± 3 %) yielded $C_m$.

### 2.3.3 Intracellular pH recordings in *Xenopus laevis* oocytes

pH microelectrodes were made from borosilicate glass capillaries without filament pulled to ~10 µM tip diameter. The lumen of the capillaries was silanized for 3 minutes in dimethylchlorosilane vapour and baked for 4 hours at 140° C. The tip of the silanized electrodes was filled with a small amount of the $H^+$ ligand cocktail containing tridodecylamine (hydrogen ionophore I, cocktail B, Sigma Aldrich) for a few hours. Prior to use, the pH electrodes were backfilled with a pH buffer solution and connected to a FD223 amplifier. The reference channel of the amplifier was connected to the voltage output signal of the TEC-05 amplifier. At the beginning of each measurement pH electrode was calibrated using standard ND96 solutions with a pH of 6.5, 7.5 and 8.5. Only electrodes with a slope ≥ 55 mV/pH unit were used for pH recordings.

### 2.3.4 Incubation experiments

The long-term effects of incubation in substrate were determined in two separate experimental settings. In a series with unpaired measurements transport parameters of the oocytes were determined only after incubation. As control, oocytes from the same batch were incubated in Barth solution, containing 5 mM manitol (pH 7.4). In the paired series, oocytes were impaled twice: before and after incubation. This enabled to compare the transport parameters of an individual oocyte before and after treatment with substrates or effectors. After the first measurement, electrodes were removed carefully and the oocytes were put into marked wells to avoid mix-ups. After incubation, oocytes were punctured and transport parameters were determined again.

If not otherwise stated, pH of the incubation medium was 7.5 and transport parameters and membrane capacitances were determined with a nearly saturating concentration of Gly-Gln (5 mM) before and/or after incubation. During incubation period the oocytes were kept at 18° C.

## 2.4 Cell culture methods

### 2.4.1 Cultivation of Caco-2 cells

Caco-2 cells were obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).* The cells were grown in minimal essential medium supplemented with 10 % fetal bovine serum, 10 % non-essential amino acids and 0.1 % gentamycin in a humidified atmosphere with 5 % $CO_2$ and a temperature of 37°C. After reaching 80 – 90 % confluency, cells, grown in 75 cm² flasks, were washed with 5 ml PBS for 5 minutes and then incubated in 3 ml trypsin until cells shrank and get separated from each other. After removing trypsin and gently knocking at the flask, cells were completely detached from the wall, dissolved in fresh medium and transferred into new flasks. For uptake experiments, cells between passages 20 and 30 were seeded at a density of $8 \times 10^5$ cells onto cell culture dishes with a diameter of 3.5 mm for 10 – 14 days post confluent. Medium was changed every second day and on the day before uptake measurement.

### 2.4.2 Preparation of cells for incubation experiments

Cell medium was removed from healthy, post confluent grown Caco-2 cells. Single dipeptides (5 mM) with/or without effectors (10 µM bestatin , 2 µM cytochalasin D, 50 µM brefeldin A) or single beta-lactam antibiotics (5 mM) were dissolved in fresh cell medium and given onto the cells. During incubation period (8 hours) Caco-2 cells were stored at 37℃.

### 2.4.3 [$^{14}$C]-Gly-Sar uptake with Caco-2 cells

Cells grown for 10 – 14 days on cell culture dishes were washed with Krebs buffer pH 6.5 for 30 s. Then, 1 ml uptake tracer pH 6.5 containing 10 µM [$^{14}$C]Gly-Sar (specific activity: 57 mCi/mmol) with or without different concentrations of cold Gly-Sar (0.01 – 20 mM) was pipetted onto the cells. Uptake measurement was performed at pH 6.5 for 10 min at room temperature. After incubation cells were washed 3 times with ice-cold Krebs buffer pH 6.5 and then dissolved in 1 ml lysis buffer, scraped off the dish with a pipette and transferred into vials containing 2 ml scintillation cocktail. Intracellular accumulation of [$^{14}$C]Gly-Sar was quantified by liquid scintillation spectroscopy.

### 2.4.4 Protein determination with Bradford Assay

Cells were washed with modified Krebs buffer (pH 6.5). After removing buffer, 1 ml sterile water was pipetted into the dish and after that cells were incubated overnight at -20° C. The following day, cells were thawed, scraped off the dish and transferred into a reaction tube. For protein extraction, the *freeze & thaw*- method was used: cells were repeatedly shock frozen and then thawed at 37° C in a water bath. Thereafter, 10 µl cell suspension was directly transferred into 200 µl *of BioRad Protein Assay*. Sample volume was filled up to 1 ml with sterile water and incubated for 15 min. Optical density was measured at a wavelength of 595.

## 2.5 Amino acid analysis

Amino acids were analyzed by ion-exchange chromatography ninhydrin-based detection with a Biochrom 30 Aminoacid-Analyzer. Quantification was performed with an external standard.

### 2.5.1 Preparation of Xenopus oocytes for amino acid analysis

Incubation medium was removed and oocytes were washed carefully with ND 96 solution. 15 - 20 oocytes were transferred into a reaction tube and centrifuged for 20 min and 12000 rpm at 15° C. Thereafter, oocytes were resuspended with 300 µl Polyvinylpyrrolidon, thoroughly mixed and again centrifuged. Supernatant was transferred into a centrifugation filter and centrifuged for 45 min at 12000 rpm and 15° C and then analyzed.

### 2.5.2 Preparation of Caco-2 cells for amino acid analysis

After incubation in substrate, cells were washed with PBS and then scraped off the dishes. After centrifugation of the cells for 10 min at 2000 rpm, supernatant was discarded, the pellet resuspended with 50 µl 1M HCl and incubated for a few minutes. For neutralisation, 50 µl 1M NaOH was added. The cells were lysed with 100 µl 10 % SSA and then incubated for 30 min on ice. Afterwards, cell suspension was centrifuged for 90 min at 12200 rpm and 4° C. The supernatant was used for amino acid analysis.

## 2.6 Statistics

### 2.6.1 Electrophysiology

Data are given as the mean ± SEM of *n* oocytes from *N* donor frogs. Statistically significant differences were determined by ANOVA and post-hoc t-tests. A p value < 0.05 was considered as significant.

### 2.6.2 Cell culture experiments

*N* is the number of separate experiments. Each uptake experiment was performed with duplicates or triplicates. *n* is the total number of dishes used in the experiments.

## 2.7 Solutions

**Krebs buffer (mM)**

| | |
|---|---|
| NaCl | 140 |
| KCl | 5.4 |
| $CaCl_2$ | 2.8 |
| $MgSO_4$ | 1 |
| HEPES | 25 (pH 7.4) |
| MES | 25 (pH 6.5) |
| glucose | 5 |

**Luria-Bertoni medium (LB-medium), pH 7.0 (mM)**

| | |
|---|---|
| NaCl | 1 % |
| Pepton | 1 % |
| yeast extract | 0.5 % |

**ND96 solution (mM)**

| | |
|---|---|
| NaCl | 96 |
| KCl | 2 |
| $MgCl_2$ | 1 |
| $CaCl_2$ | 1.8 |
| HEPES | 5 |

**ND96 solution, calcium free (mM)**

| | |
|---|---|
| NaCl | 96 |
| KCl | 2 |
| $MgCl_2$ | 1 |
| HEPES | 5 |

**pH buffer solution, pH 7.0 (mM)**

| | |
|---|---|
| KCl | 500 |
| NaH2PO4 | 64.7 |
| Na2HPO4 | 85.3 |

**Phosphat buffer solution (PBS), pH 7.4 (mM)**

| | |
|---|---|
| NaCl | 136 |
| $Na_2HPO_4$ x 2 $H_2O$ | 8.1 |
| KCl | 2.7 |
| $KH_2PO_4$ | 1.5 |

## 2.8 Chemicals and other materials

| | |
|---|---|
| actinomycin D | Sigma-Aldrich, Deisenhofen |
| agarose | Roth, Karlsruhe |
| Alexa fluor 488 goat anti-mouse IgG | Molecular Probes (Invitrogen), Groningen, Netherlands |
| alpha-methyl-di-pyranoside (α-MDG) | Sigma-Aldrich, Deisenhofen |
| 3-aminobenzoic acid ethyl ester (MS-222) | Sigma-Aldrich, Deisenhofen |
| ANTI-FLAG® M2 monoclonal antibody from mouse | Sigma-Aldrich, Deisenhofen |
| *BioRad Protein Assay* | BioRad, München |
| bisindolylmaleimide | Sigma-Aldrich, Deisenhofen |
| borosilicate capillaries | World Precision instruments, Berlin |
| bovine serum albumin | Sigma-Aldrich, Deisenhofen |
| brefeldin A | Sigma-Aldrich, Deisenhofen |
| cefadroxil | Sigma-Aldrich, Deisenhofen |
| cell culture flasks (75 $cm^2$) | greiner bio-one, Frickenhausen, |
| cell culture dishes, Falcon easy grip | Becton Dickinson GmbH, Heidelberg |
| collagenase A | Roche Diagnostics, Mannheim |
| cycloheximide | Calbiochem, Schwalbach |
| cytochalasin D | Sigma-Aldrich, Deisenhofen |
| dimethylchlorosilane | Serva, Heidelberg |
| *escherichia coli* strain Top 10 F' | Invitrogen, Groningen, Netherlands |

| | |
|---|---|
| *flexi*$^{TM}$ kit for DNA preparation | Amersham Biosciences, Freiburg |
| fetal bovine serum (FBS) Gold | PAA Laboratories, Colbe |
| frog pellets | Kähler, Hamburg |
| gentamycin | Alexis Biochemicals, Grünberg |
| gentamycin solution for cell culture | Gibco, Karlsruhe |
| glycine | Sigma-Aldrich, Deisenhofen |
| glycyl-sarcosine | Sigma-Aldrich, Deisenhofen |
| hydrogen ionophore I, cocktail B | Sigma-Aldrich, Deisenhofen |
| isopropanol | Roth, Karlsruhe |
| minimal essential medium (MEM) with Earle's salts and L-glutamine | PAA Laboratories, Colbe |
| *mMessage mMachine – high yield capped RNA transcription kit* | Ambion, Austin, Texas, USA |
| non-essential amino acids | PAA Laboratories, Colbe |
| phorbol-12,13-dibutyrate | Sigma-Aldrich, Deisenhofen |
| radiolabelled $^{14}$C-glycyl-sarcosine ($^{14}$C-Gly-Sar) | Amersham Biosciences, Freiburg |
| Roti® Phenol/Chloroform | Roth, Karlsruhe |
| scintillation cocktail: *Roti-Szint eco plus* | Roth, Karlsruhe |
| Shrimp alkaline phosphatase (SAP) | Boehringer Mannheim GmbH, Mannheim |
| sodium pyruvate | Sigma-Aldrich, Deisenhofen |
| T4 DNA ligase | Invitrogen, Groningen, Netherlands |
| *Thermo Sequenase Cy5 Dye Terminator* kit | Amersham Biosciences, Freiburg |
| trypsin | PAA Laboratories, Colbe |
| *QIAquick Gel Extraction* kit | Qiagen GmbH, Hilden, |
| *QuikChange Site-directed Mutagenesis* kit | stratagene, Amsterdam, Netherlands |

Glycyl-glutamine was a generous gift from Degussa-Rexim.

Restriction enzymes were purchased from MBI Fermentas(St. Leon-Rot) or from NEB (Frankfurt).
Oligonucleotides were obtained from MWG Biotech AG, Ebersberg.
All other chemicals were purchased from Sigma-Aldrich (Deisenheim), Roth (Karlsruhe) or Merck (Darmstadt).

## 2.9 Technical equipment and software

| | |
|---|---|
| ALFexpress$^{TM}$ DNA sequencer | Amersham, Freiburg |
| ALFwin Sequence analyzer 2.1 software | Amersham, Freiburg |
| BIOphotometer | Eppendorf, Köln |
| CellWorks software | npi electronic, Tamm |
| counter 1450 Microbeta Trilux | EGRG Wallace, Turku, Finland |
| DNA electrophoresis chamber | BioRad, München |
| Drummond nanoinjector | World Precision Instruments, Berlin |
| electrode puller | Zeitz Instrumente GmbH, München |
| FD223 amplifier | World Precision Instruments, Berlin |
| TEC-05 amplifier | npi electronic, Tamm |

# 3 RESULTS: ELECTROPHYSIOLOGICAL EXPERIMENTS

## 3.1 Insertion of rPept1 proteins into the plasma membrane resulted in an increase in membrane surface area

### 3.1.1 Membrane capacitance of non-injected, water-injected and rPept1-expressing oocytes

To determine whether the expression of rPept1 in the oocytes membrane is accompanied by an increase in membrane surface area, transport currents and membrane capacitances of non-injected, water-injected and rPept1-expressing oocytes were measured as described in 2.3.1 and 2.3.2. The results are shown in Tab. 3.1.
As expected, both non-injected and water-injected oocytes did not generate any transport currents, confirming that *Xenopus* oocytes naturally do not express endogenous peptide transporters. The mean membrane capacitances of these two experimental groups were 221 ± 11 nF and 224 ± 5 nF, respectively, which was not significantly different.
In contrast, expression of rPept1 resulted in a significant increase in membrane capacitance to 301 ± 6 nF ($p < 0.01$) with a mean transport current of -428 ± 32 nA.

To observe, whether the increase of membrane capacitance resulted from an increased size of the oocyte, the diameters of non-injected (NI), water-injected (WI) and rPept1-expressing (Pept1) oocytes were determined with an ocular micrometer. The data shown in Tab. 3.1 indicate no differences in diameter between all three experimental groups.

### 3.1.2 Differences in surface amplification factor

Since the diameters of rPept1-expressing and water-injected oocytes were not significantly different, the increase in membrane capacitance must have been the result of an increase in surface membrane complexity. The membrane surface of *Xenopus* oocytes is not smooth, but consists of numerous microvilli (Zampighi et al., 1995), thus resulting in an increase in membrane surface area as compared to a smooth globe. In a next attempt it was investigated whether the expression of rPept1 in the membrane surface was accompanied with an increase in membrane surface amplification.

From the capacitance and diameter data an amplification factor of rPept1-expressing oocytes was calculated which was 9 x higher than the surface of a smooth globe with the same diameter. In water- and non-injected oocytes this factor was only 7 x, which is significantly lower as compared to rPept1-expressing oocytes ($p < 0.01$), suggesting that the insertion of rPept1 proteins into the oocyte membrane is accompanied with an increase in membrane surface complexity. The data are shown in Tab. 3.1.

| experimental groups | transport currents [nA] | membrane capacitances [nF] | Ø [mm] | surface amplification factor |
|---|---|---|---|---|
| **NI** (N/n=3/10) | 0 | 221 ± 11 | 1.00 ± 0.03 | 7.04 ± 0.44 |
| **WI** (N/n=5/24) | 0 | 224 ± 5 | 1.04 ± 0.02 | 6.78 ± 0.31 |
| **Pept1** (N/n=5/34) | -428 ± 32 | 301 ± 6** | 1.04 ± 0.02 | 9.01 ± 0.33** |

**Tab. 3.1:** Transport currents, membrane capacitances, diameter and surface amplification factor of non-injected (NI), water-injected (WI) and rPept1-expressing oocytes
**) Comparison between water-injected and rPept1-expressing oocytes ($p < 0.01$)

## 3.2 Effects of a prolonged exposure to substrate on transport currents and membrane capacitances of rPept1-expressing oocytes

### 3.2.1 Exposure of *Xenopus* oocytes to low concentrations of dipeptides

Previous studies have shown that prolonged exposure (24 hours, respectively 3 days) of Caco-2 cells to the dipeptide glycyl-sarcosine (Gly-Sar) resulted in an increase in $^{14}$C-Gly-Sar uptake due to an increase in levels of Pept1 protein and mRNA (Thamotharan et al., 1998; Walker et al., 1998). To investigate whether activity of rPept1 overexpressed in *Xenopus* oocytes is regulated in a similar way, after injection of rPept1 cRNA oocytes were exposed to different low concentrations of the dipeptide glycyl-glutamine (Gly-Gln: 0, 20, 30, 40, 50 µM) for 3 days. During the incubation period, the dipeptide-containing medium was changed daily. After 3 days, oocytes were transferred into a dipeptide-free medium and peptide-induced transport currents were measured by TEVC at a nearly saturating substrate concentration (5 mM Gly-Gln). As shown in Fig. 3.2.1, in contrast to the effects observed in Caco-2 cells, transport currents of rPept1-expressing oocytes did not increase but decreased in a concentration dependent manner.

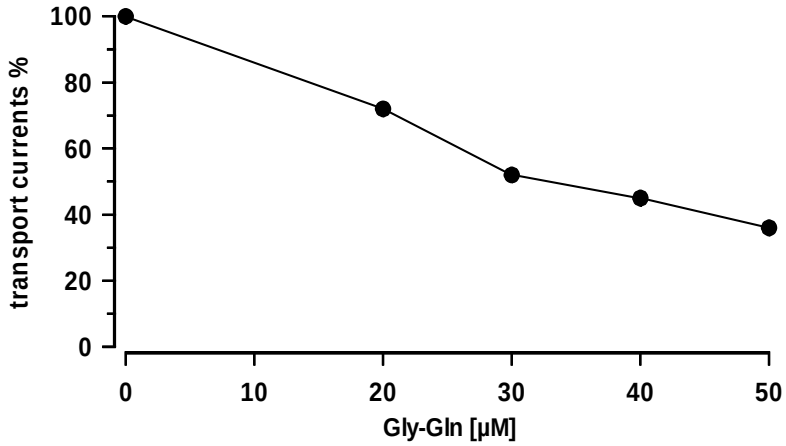


**Fig. 3.2.1:** Exposure of *X.* oocytes directly after injection of rPept1-cRNA for 3 days to 0, 20, 30, 40 or 50 µM Gly-Gln decreased transport currents of rPept1 in a concentration dependent manner.

### 3.2.2 Exposure of *Xenopus* oocytes for 4 hours to a saturating concentration of Gly-Gln (unpaired experiments)

To investigate, whether a shorter incubation period but a higher dipeptide concentration also diminishes transport activity of rPept1, rPept1-expressing oocytes were 3 days after cRNA injection –at the time point with the highest protein expression levels- exposed to a nearly saturating concentration of Gly-Gln (5 mM) for 4 hours. In parallel, three other groups of oocytes from the same batch served as controls and were treated for the same time as follows: rPept1-expressing oocytes were exposed to 5 mM mannitol (for compensation of osmolarity increase of the dipeptide solution) and water-injected oocytes either to 5 mM Gly-Gln or to 5 mM mannitol. Transport currents and membrane capacitances were measured only after incubation and compared to that of the control groups.

Tab. 3.2.2 shows the data of water-injected and rPept1-expressing oocytes determined after exposure to 5 mM Gly-Gln or control solution.

As expected, both water-injected oocytes exposed to a dipeptide-containing solution (WI+) as well as to the mannitol containing solution (WI-) did not exhibit any transport current. Their membrane capacitances were not different and were significantly lower as that of rPept1-expressing oocytes exposed to control solution.

Distinct differences were observed between rPept-expressing oocytes incubated in control solution (Pept1-) and dipeptide-containing solution (Pept1+). After incubation in the mannitol-containing solution control oocytes had a mean transport current of -486 ± 57 nA and a membrane capacitance of 307 ± 12 nF. In contrast, the transport current of oocytes exposed to Gly-Gln decreased largely to -124 ± 10 nA and membrane capacitance did not significantly differ from that of water-injected oocytes.

The macroscopic diameter of all four experimental groups did not vary considerably. The calculated surface amplification factor of rPept1-expressing control oocytes was larger than that of water-injected oocytes. Remarkably, surface amplification factors of rPept1-expressing oocytes exposed to Gly-Gln and water-injected oocytes were similar.

| experimental groups | transport current [nA] | membrane capacitance [nF] | Ø [mm] | Surface amplification factor |
|---|---|---|---|---|
| WI- (N=2/6) | - | 214 ±10 | 1.01 ± 0.03 | 6.79 ± 0.45 |
| WI+ (N=2/6) | - | 208 ± 6 | 1.04 ± 0.03 | 6.24 ± 0.41 |
| Pept1- (N/n=2/13) | -486 ± 57 | 307 ± 13 $^{++}$ | 1.07 ± 0.03 | 8.58 ± 0.40 $^{+}$ |
| Pept1+ (N/n=2/11) | -124 ± 10 ** | 225 ± 8 ** | 1.11 ± 0. 02 | 5.88 ± 0.35 ** |

**Tab. 3.2.2:** Transport currents, membrane capacitances, macroscopic diameter, surface amplification factor of rPept1-expressing and water-injected (WI) oocytes after exposure to 5 mM Gly-Gln (+) or 5 mM mannitol (-)
$^{+/++}$) comparison between water-injected and Pept1-expressing oocytes after exposure to 5 mM mannitol
**) comparison between Pept1-expressing oocytes after exposure to mannitol (Pept1-) and dipeptide-containing solution (Pept1+)

Fig. 3.1 shows the relationship of the membrane capacitance as a function of the peptide-induced transport currents after exposure to substrate or control solution. For this graphical presentation an additional set of oocytes were measured. The linear regression fitted to the data point had a slope of -0.109 nF/nA and an intercept at 238 nF, corresponding to an increase in membrane capacitance by 10.9 nF per -100 nA of inward current.

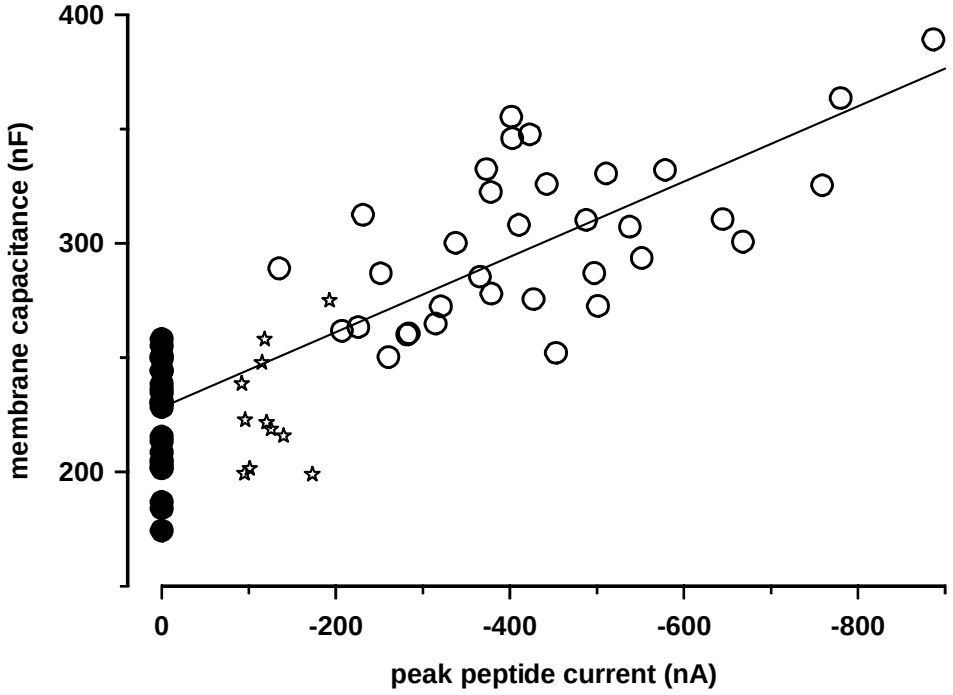


**Fig. 3.1:** Membrane capacitances as a function of the transport currents of water-injected (●, N/n=5/24) and of rPept1-expressing oocytes after exposure to Gly-Gln (☆, N/n=3/11) or mannitol-containing solution (○, N/n=5/34) for 4 hours

So far, the results shown in Fig. 3.1 indicate, that

a) the expression of rPept1 in *Xenopus* oocytes is accompanied with an increase in membrane capacitance, respectively membrane surface complexity
b) a prolonged exposure to substrate diminishes transport currents of rPept1 in parallel with a decrease in membrane capacitance
c) the membrane capacitance of rPept1-expressing oocytes after exposure to a dipeptide-containing solution is reduced to the level of water-injected oocytes

### 3.2.3 Effects on membrane capacitance and transport current after 2 hours and 4 hours exposure to Gly-Gln (paired experiments)

Although the previous series clearly demonstrated that incubation in Gly-Gln-containing solution markedly reduces transport current and membrane capacitance, the changes for individual oocytes were not determined, since the measurements were unpaired.
Therefore, all oocytes in the following experiments were punctured twice: before and after exposure to 5 mM Gly-Gln or control solution (5 mM mannitol). A detailed protocol of this experiment is found in section 2.3.4.

The first two groups of oocytes were incubated for only 2 hours. The data in Tab. 3.2.3 and Fig. 3.2 show that transport currents of the dipeptide-exposed oocytes decreased significantly by 59 ± 8 % while transport currents of the control group increased after 2 hours. In spite of these different changes membrane capacitance of these groups remained almost unchanged and decreased only about 8 % and 4 % respectively.

The third and fourth groups of oocytes were incubated for 4 hours under otherwise identical experimental conditions. The results are also shown in Tab. 3.2.3 and Fig 3.2.

Transport currents of control oocytes remained unchanged after 4 hours incubation. Their membrane capacitance decreased only slightly by 10 ± 2 %. Exposure to substrate reduced transport currents by 69 ± 3 % (i.e. about 24 % of the transporters still present at the membrane surface after 2 hours were removed between 2 and 4 hours) as well as membrane capacitances by 34 ± 4 % in rPept1-expressing oocytes.

| experimental groups | transport current [nA] | | | membrane capacitance [nF] | | | current density [nA/nF] | | |
|---|---|---|---|---|---|---|---|---|---|
| | before | after | %[1] | before | after | %[1] | before | after | %[1] |
| control 2h (N/n=2/6) | -484 ± 66 | -605 ± 62 | +33 ± 18 | 322 ± 5 | 308 ± 4* | -4 ± 1 | -1.5 ± 0.2 | -2.0 ± 0.2** | +39 ± 18 |
| Gly-Gln 2h (N/n=2/7) | -575 ± 63 | -231 ± 21** | -59 ± 8 | 337 ± 9 | 311 ± 9** | -8 ± 1 | -1.7 ± 0.2 | -0.7 ± 0.0** | -55 ± 3 |
| Control 4h (N/n=2/6) | -601 ± 75 | -596 ± 75 | -4 ± 4 | 375 ± 23 | 339 ± 23* | -10 ± 2 | -1.3 ± 0.1 | -1.6 ± 0.0** | +15 ± 2 |
| Gly-Gln 4h (N/n=2/9) | -505 ± 49 | -149 ± 17** | -69 ± 3 | 342 ± 6 | 224 ± 3** | -34 ± 4 | -1.5 ± 0.1 | -0.7 ± 0.2** | -53 ± 4 |

**Tab. 3.2.3:** Transport currents, membrane capacitances and current densities before and after exposure to 5 mM Gly-Gln or 5 mM mannitol for 2 or 4 hours
[1]) Difference in % between before and after incubation
*/** Comparison between before and after incubation of the same experimental group

In addition, Tab. 3.2.3 and Fig. 3.2 show the current density before and after exposure to Gly-Gln and control solution. The current density [nA/nF] is the relation between transport currents [nA] and membrane capacitances [nF]. Assuming that the turnover rate of Pept1 before and after incubation remains unchanged, the transport density is a measure of the transporter density per membrane unit of area.

The current density of the control oocytes increased significantly after 2 and 4 hours exposure to mannitol (+39 ±18 % , +15 ± 2 %).

Exposure of rPept1-expressing oocytes for 2 hours in Gly-Gln resulted in a large reduction in mean transport currents with only a small change in the membrane capacitance and in turn, in a decline in current density. After 4 hours incubation, transport currents decreased to a larger extent than the membrane capacitance, leading to a decline in current density. Importantly, the decrease of current density of rPept1-expressing oocytes treated with Gly-Gln was equal after 2 hours and 4 hours which is the result of a larger decline in transport currents within the first 2 hours of incubation followed by a larger decrease in capacitance within the remaining 2 hours of incubation.

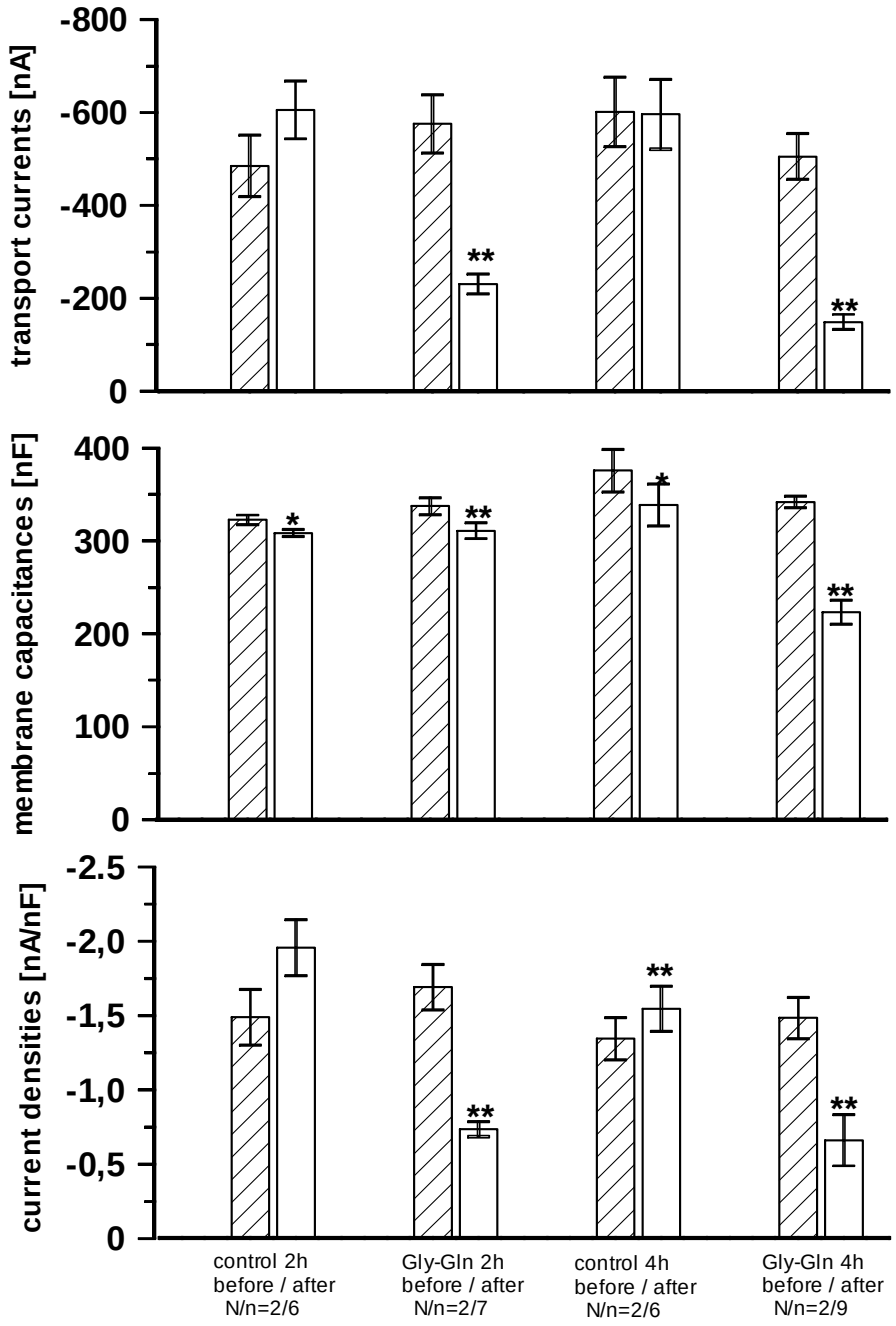


**Fig. 3.2:** Transport currents, membrane capacitances and current densities of rPept1-expressing oocytes before and after 2 hours, respectively 4 hours exposure to control- or dipeptide-containing solution
*/** Comparison between before and after incubation of the same experimental group

### 3.2.4 Determination of the maximal transport velocity ($I_{max}$) and the Michaelis-Menten constant ($K_m$) before and after exposure to substrate

The large decrease of the dipeptide-induced current in response to incubation in Gly-Gln suggested a decrease of the maximal transport rate of Pept1. Theoretically, however, a largely decreased substrate affinity could have caused the same effect. Although this did not seem very probable the maximal transport velocity ($I_{max}$) and the Michaelis-Menten constant ($K_m$) were calculated to exclude the latter possibility.

The Michaelis-Menten constant ($K_m$) is the substrate concentration at which the rate of transport activity is half its maximum. It is also a measure of the substrate affinity whereas a low $K_m$-value indicates a high substrate affinity and vice versa.
The maximal velocity ($I_{max}$) can directly be measured at very high substrate concentrations and can be calculated from measurements at different substrate concentrations according to the Michaelis-Menten equation.

To calculate the above parameters, peptide-induced transport currents were measured before and after exposure to Gly-Gln with three concentrations of Gly-Gln (0.5, 1.5, 5.0 mM). In parallel, control experiments using oocytes from the same frog were performed (incubation for 4 hours in 5 mM mannitol).

Substrate affinities were calculated by perfusing the oocytes alternately with ND96 and substrate-containing solution, starting with the highest concentration. IV curves were calculated in the range between –160 and –40 mV.

Figs. 3.3 and 3.4 show that the potential dependency of $I_{max}$ (normalized to the value measured at –160 mV was not different between control experiments (Fig. 3.3) and after exposure to Gly-Gln (Fig. 3.4). The substrate affinity decreased slightly in oocytes exposed to mannitol, but increased after exposure to Gly-Gln.

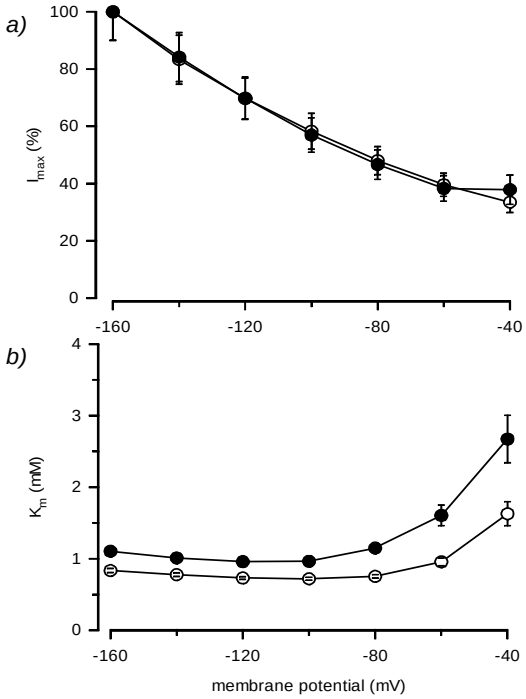


**Fig 3.3: a)** $I_{max}$ (%) and **b)** $K_m$ (mM) as a function of the membrane potential in rPept1-expressing oocytes before and after exposure to control solution (mannitol-containing) for 4 hours (N/n = 2/11; ○ before incubation / ● after incubation)

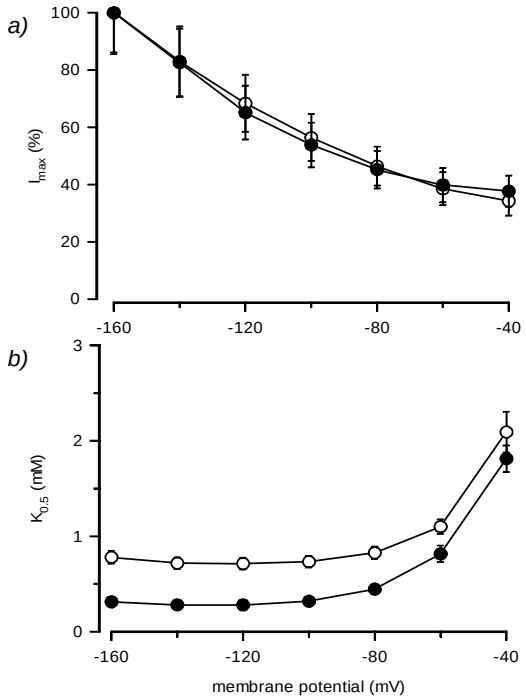


**Fig 3.4: a)** $I_{max}$ (%) and **b)** $K_m$ (mM) as a function of the membrane potential in rPept1-expressing oocytes before and after exposure to Gly-Gln for 4 hours. (N/n = 2/6; ○ before incubation / ● after incubation)

## 3.3 Effects of a prolonged substrate exposure on membrane surface expression of rPept1

Decreased transport activity may be the result of a reduced transport (turnover) rate of individual transporters or a reduced abundance of transporters in the membrane. A reduced membrane area suggested the second possibility. In the following series of experiments optical methods were used to quantify the amount of transporters in the membrane and thus to verify the observations made by electrophysiology.

Endocytosis is a process whereby proteins or other material is absorbed by the plasma membrane of a cell and translocated to intracellular compartments. The fate of the proteins that undergo endocytosis varies: a part of them are stored in vesicles to allow redistribution to the membrane surface, whereas the other part is packed in lysosomes for degradation.
A substrate induced endocytosis process in *Xenopus* oocytes has been first observed in the dopamine transporter DAT (Gulley et al., 2002). Treatment of DAT-expressing oocytes with low micromolar concentrations of the DAT substrates dopamine and *d*-amphetamine (AMPH) reduced transport currents markedly. Similar observations have already been made two years ago by Saunders et al (2000), indicating that treatment of human embryonic kidney HEK 293 cells with AMPH resulted in a decrease of DAT-induced transport currents and $^{3}$[H]DA uptake. Immunofluorescence detection showed an increase in intracellular DAT as a result of a substrate induced removal of dopamine transporters from the membrane surface. Due to these observations it might be possible that transport activity of Pept1 is regulated in a similar way.

### 3.3.1 Reduction in membrane expression of FLAG-labelled rPept1 proteins after exposure to substrate

To investigate whether the decrease in transport current and membrane capacitance after exposure to Gly-Gln is due to an endocytosis of a part of the peptide transporters from the membrane surface to intracellular compartments, a FLAG-epitope was inserted into the large loop of rPept1 sequence enabling the detection of transporters actually located at the membrane surface.

Oocytes expressing the FLAG-tagged peptide transporters were exposed either to Gly-Gln (5 mM) or to control solution, containing 5 mM mannitol, for 4 hours. Thereafter, FLAG-labelled transporter proteins present at the membrane surface were immunolabelled and detected by fluorescence microscopy. The average fluorescence intensity of substrate-exposed oocytes was 3765 ± 340 (arbitrary units; N/n=3/25) which was about 38 % lower ($p < 0.01$) than that of control oocytes (6056 ± 340; N/n=3/25). This result is in accordance with the reduction in membrane capacitance by about 34 % observed after an incubation period of 4 hours.

### 3.3.2 The role of the cytoskeleton in the substrate-induced decline in transport activity and membrane capacitance of rPept1-expressing oocytes

A prerequisite for endo- an exocytosis is an intact cytoskeleton since it is significantly involved in these processes. As a consequence, disrupture of the cytoskeleton eliminates trafficking processes of the cell (Verrey et al., 1995; Najimi et al., 2002).
Hence, to further prove the possibility that endocytosis is the mechanism of the substrate-induced reduction in transport activity and membrane capacitance in rPept1-expressing oocytes, the normal function of the cytoskeleton in oocytes was blocked by application of cytochalasin D (CD), an inhibitor of actin polymerization.

For this experiment, rPept1-expressing oocytes were initially exposed to 5 mM Gly-Gln for 1 – 1 ½ hours and then incubated simultaneously in Gly-Gln and 10 µM CD for further 2 ½ - 3 hours. A longer incubation period in CD was not possible because of its toxic effect on the integrity of the oocyte membrane.

Exposure of rPept1-expressing oocytes to Gly-Gln in the absence of CD reduced transport currents by about 69 % and membrane capacitances by about 34 % (Tab. 3.2.3).
In the simultaneous presence of Gly-Gln and CD transport currents decreased from -337 ± 30 to -233 ± 27 nA (by 30 ± 7 %) and membrane capacitance from 316 ± 9 nF to 257 ± 8 nF (by 19 ± 2 %).
The reduction in transport current and membrane capacitance in the presence of CD is significantly lower than after incubation in Gly-Gln alone ($p<0.01$ / $p<0.05$). Thus, inactivation of the cytoskeleton partially blocked the substrate-induced changes in transport activity and membrane capacitance.

## 3.4 Examining the substrate specifity: prolonged exposure of rPept1-expressing oocytes to the beta-lactam antibiotic cefadroxil

To get more insight, whether the decrease in transport activity and membrane capacitance observed after exposure to substrate is due to an intracellular accumulation of dipeptides or amino acids, similar experiments were performed with the beta-lactam antibiotic cefadroxil, a non-peptide substrate of Pept1 (Ki = 7.2 ± 0.8 mM / Bretschneider et al., 1999) that is, in contrast to Gly-Gln, not hydrolysed. rPept1-expressing oocytes were exposed to 20 mM cefadroxil for 5 hours. The higher concentration was selected due to the lower affinity of cefadroxil as compared to Gly-Gln. Again, control experiments using 20 mM mannitol were performed in parallel. The peptide-induced transport currents and membrane capacitances were measured before and after incubation.
The transport currents before incubation were -452 ± 46 nA and declined to -258 ± 57 nA (to 56.5 ± 4.3 %; $p<0.01$) after incubation (N/n = 2/13). The average membrane capacitance decreased from 302 ± 7 nF to 180 ± 7 nF (to 59.9 ± 2.2 %; $p<0.01$), which is similar to the effects caused by exposure to Gly-Gln for 4 hours.

## 3.5 Effects of substrate binding without transport

The previous results showed that prolonged exposure of oocytes to the dipeptide Gly-Gln or the beta-lactam antibiotic cefadroxil resulted in a profound decrease in transport activity and membrane capacitance of rPept1. To investigate whether alone the binding of substrate without its transport is sufficient to reduce Pept1 activity, oocytes were incubated in the high-affinity inhibitor of Pept1 Lys-[Z($NO_2$)]-Pro ($K_i$ = 29 ± 4 µM; Knütter et al., 2001) that occupies for the substrate-binding site but is not transported itself.

rPept1-expressing oocytes were exposed to 5 mM Lys-[Z($NO_2$)]-Pro for 4 hours. The peptide-induced transport currents and membrane capacitances were measured before and after incubation with 5 mM Gly-Gln.

| experimental group | Transport currents [nA] | | | Membrane capacitances [nF] | | |
|---|---|---|---|---|---|---|
| | before | after | % | before | after | % |
| Lys-[Z($NO_2$)]-Pro | -426±59 | -383 ±35 | 100±6 | 346 ± 7 | 280 ± 10** | 81 ±2 |
| Control[1)] | -296 ±58 | -387±60* | 137±11 | 321 ± 13 | 298 ± 6* | 93±2 |

**Tab 3.5:** Transport currents and membrane capacitances before and after 4 hours exposure of rPept1-expressing oocytes to the inhibitor Lys-[Z($NO_2$)]-Pro (N/n=2/14) or to control solution
[1]) data of control oocytes taken from section 3.2.3 (control group 4 h)
**) compared to the data of the same experimental group before incubation

The data in Tab. 3.5 indicate that transport currents remained unchanged after exposure of oocytes to Lys-[Z($NO_2$)]-Pro for 4 hours, whereas transport currents of control oocytes increased by about 37 %.

Although the average membrane capacitance of rPept1-expressing oocytes exposed to Lys-[Z($NO_2$)]-Pro decreased significantly stronger (to 81 ± 2 %; $p < 0.05$) than that of the control group (to 93 ± 2 %), this reduction was only slightly more than the half of the change observed after 4 hours exposure to Gly-Gln.

To summarize, these data clearly show that not substrate binding per se but active transport of substrate into the oocytes is necessary to alter transport activity of Pept1

## 3.6 Qualitative analysis of intracellular amino acids and dipeptides after exposure of rPept1-expressing oocytes to Gly-Gln

The following experiment was performed to investigate the fate of the dipeptides transported into the oocyte during a 4 hours incubation period.

To observe whether the transported dipeptides were accumulated intracellularly in intact form or were hydrolysed to free amino acids, a qualitative amino acid analysis of the cytosol of rPept1-expressing oocytes after a 4 hours incubation period with Gly-Gln was performed. Detailed description of this procedure is found in section 2.5.

Fig. 3.6 a) shows the standard retention time of Gly-Gln (72.59 min), i.e. the time when Gly-Gln elutes. For this purpose 5 mM Gly-Gln diluted in Barth-solution (pH 7.4) were prepared and taken as reference value.

The cytosol of control oocytes (Fig. 3.6 b) as well as dipeptide-treated rPept1-expressing oocytes (Fig. 3.6 c) contained small, but measurable amounts of aspartic acid and

glutamic acid. Surprisingly, in oocytes exposed to Gly-Gln no significant amounts of the dipeptide but the presence of the free amino acids glycine and glutamine was observed (retention time of glycine: 59.30 min; retention time of glutamine: 45.62 min). In contrast, neither of these two amino acids were found in control oocytes, suggesting that prolonged incubation resulted in a hydrolysis of the dipeptide Gly-Gln to free amino acids.

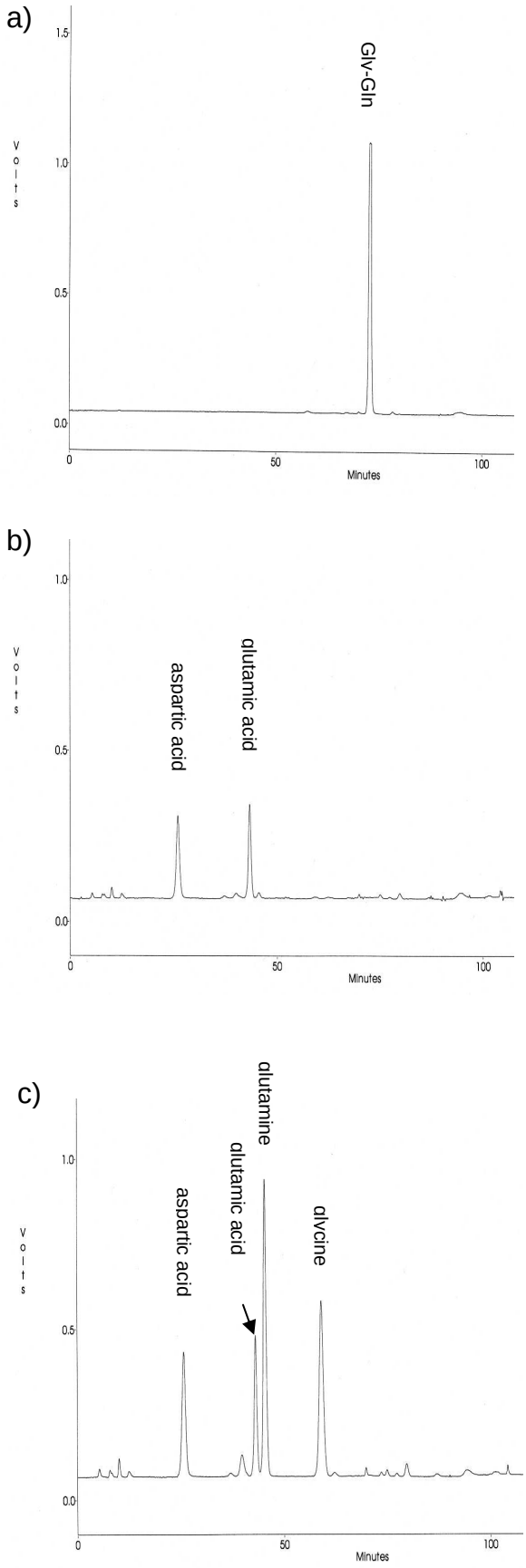


Fig. **3.6: a)** Chromatograms of Gly-Gln standard and of the intracellular content of amino acids of rPept1-expressing oocytes exposed to **b)** control solution and **c)** 5 mM Gly-Gln

## 3.7 Inhibition of dipeptide hydrolysis with bestatin

The amino acid analysis of the cytosol of oocytes (see 3.6) indicated that the dipeptide Gly-Gln taken up during the 4 hours of incubation has been completely hydrolysed to free amino acids. Consequently, the hypothesis that an intracellular accumulation of dipeptide triggers the downregulation of rPept1 activity turned out to be false. Moreover, exposure of oocytes to cefadroxil, which is not hydrolysed intracellularly, had similar negative effects on the transport activity of rPept1 as had Gly-Gln. In consideration of these results the next experiments aimed at the inhibition of intracellular hydrolysis of Gly-Gln by simultaneous application of the metallo-protease inhibitor bestatin, which inhibits aminopeptidases but not carboxypeptidases.

### 3.7.1 Short-term exposure of Pept1-expressing oocytes to bestatin

Preliminary tests were performed to verify that the presence of bestatin in a Gly-Gln-containg solution inhibits hydrolysis of the dipeptide.
For this purpose rPept1-expressing oocytes were exposed to 0.1 mM bestatin for at least 1 hour. Afterwards, oocytes were initially superfused with ND96 solution containing 0.1 mM bestatin without Gly-Gln until reaching stable measuring conditions. Then, 5 mM Gly-Gln supplemented with 0.1 mM bestatin was applied and maximal inward currents recorded, followed by perfusing the oocytes with standard ND96 solution to wash out extracellular Gly-Gln. IV-curves were recorded in the presence of Gly-Gln, when dipeptide-induced inward currents reached its maximum (phase 1), after washing out extracellular Gly-Gln, before reversion of transport currents in outward direction (phase 2) and when maximal outward-directed were reached (phase 3, all observations at –60 mV).

The I/V curves shown in Fig 3.7.1 demonstrate that in phase 1 (o) and phase 2 (☆) outward directed transport currents were only generated at positive membrane potentials. In phase 3 (●), however, large outward currents were generated at positive and negative membrane potentials, whereas inward-directed currents were probably only induced at membrane potentials more negative than –160 mV. The appearance of outward currents at negative membrane potential suggests the presence of high concentrations of unhydrolized Gly-Gln in the oocyte, i.e. that bestatin prevented hydrolysis of Gly-Gln.

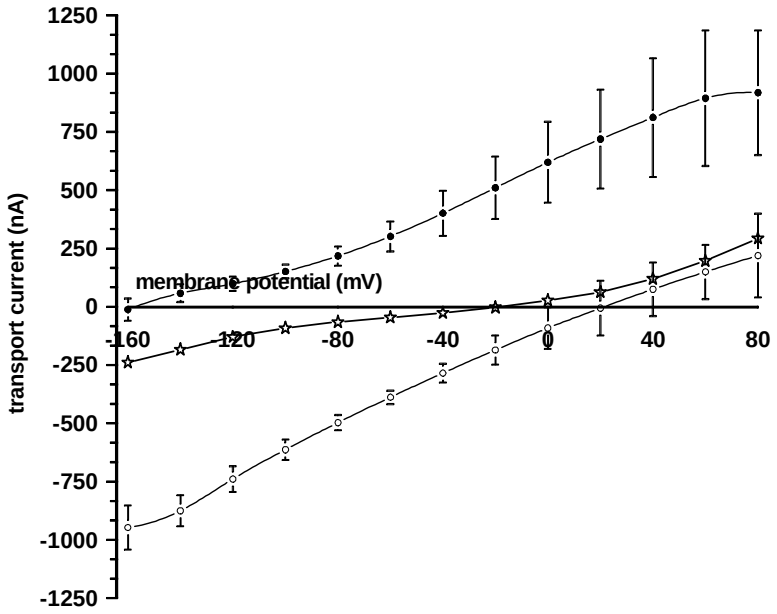


**Fig. 3.7.1:** IV-curves after pre-exposure of rPept1-expressing oocytes to 0.1 mM bestatin. ° phase 1: in the presence of 5 mM Gly-Gln, ☆ phase 2: before transport currents were reversed in outward direction at -60 mV • phase 3: when maximal outward-directed currents at –60 mV were reached (N/n = 3/4)

### 3.7.2 Long-term exposure of rPept1-expressing oocytes to bestatin

The next set of experiments was performed to investigate what happens when oocytes are exposed to Gly-Gln for 4 hours in the simultaneous presence of bestatin to inhibit intracellular hydrolysis.

rPept1-expressing oocytes were exposed to 0.1 mM bestatin for 1 hour. Then, oocytes were transferred to a dipeptide-containing solution (5 mM Gly-Gln) supplemented with 0.1 mM bestatin for 5 hours. One control group was exposed to 0.1 mM bestatin without Gly-Gln (negative control), the second control group was incubated in 20 mM cefadroxil without bestatin (positive group) for the same time. Transport parameters were measured before and after incubation with 5 mM Gly-Gln or 20 mM cefadroxil (positive control group).

The results are shown in Tab. 3.7.2.

The transport activity of the negative control group increased slightly, but significantly. As expected, transport currents and membrane capacitances of rPept1-expressing oocytes

decreased markedly after exposure to cefadroxil. Simultaneous exposure to bestatin and Gly-Gln resulted in a noticeable decline in membrane capacitance in parallel with a significant increase in mean transport currents.

| | transport currents (nA) | | membrane capacitance (nF) | |
|---|---|---|---|---|
| | before | after | before | after |
| negative control group (bestatin) N/n = 1/6 | --256 ± 16 | -281 ± 13* | 284 ± 6 | 292 ± 7* |
| positive control (cefadroxil) N/n = 1/6 | -229 ± 11 | -119 ± 8** | 312 ± 5 | 274 ± 7** |
| bestatin + Gly-Gln N/n = 2/15 | -320 ± 17 | -409 ± 20** | 317 ± 5 | 287 ± 5** |
| Gly-Gln[1)] N/n = 2/9 | -505 ± 49 | -149 ± 17** | 342 ± 6 | 224 ± 3** |

**Tab. 3.7.2:** Transport currents and membrane capacitances before and after exposure to bestatin (negative control group), cefadroxil (positive control group), Gly-Gln and simultaneous incubation in bestatin + Gly-Gln
*) compared to the data before incubation of the same group
[1]) data taken from Tab. 3.2.3

## 3.8 Search for the mechanism involved in the substrate-induced reduction of transport activity and membrane surface expression of rPept1

The results of the chapters 3.4 and 3.5 indicate that the substrate-induced reduction in transport activity and membrane surface expression of rPept1 is not specific for the dipeptide Gly-Gln, but also occurred when oocytes were exposed to the beta-lactam antibiotic cefadroxil and that substrate has to be transported into the oocyte to alter transport parameters of rPept1.
The aim of the following investigations was to search for the mechanism responsible for the substrate-induced endocytosis of peptide transporters.

### 3.8.1 Involvement of proteinkinase C in regulation of transport activity and membrane surface expression of rPept1

Detailed studies have indicated a role of proteinkinase C (PKC) in the regulation of transport activity and surface expression of many transporters and channels. Acute treatment of *X.* oocytes expressing SGLT1 or the mouse retinal taurine transporter with *sn*-1,2-dioctanoglygerol (an activator of PKC) resulted in a reduction in maximal transport rate by about 50 % (Hirsch et al., 1996; Loo et al., 1996). Studies on the $Na^{(+)}$/dicarboxylate cotransporter, which is responsible for the reabsorbtion of Krebs cycle intermediates such as citrate and succinate showed that activation of PKC with phorbol 12-myristate, 13-acetate (PMA) inhibited succinate transport in *X. oocytes* up to 95 % (Pajor and Sun, 1999).

On the basis of these investigations, the possible involvement of PKC in regulation of Pept1 activity was tested by exposure of rPept1-expressing oocytes to the phorbolester phorbol-12,13-dibutyrate (PDBu; 1 µM), a specific activator of PKC.

Exposure of rPept1-expressing oocytes for only 1 hour in PDBu (Tab. 3.8.1 and Fig. 3.8.1) resulted in a reduction of both transport currents (50 ± 3 %) and membrane capacitance (55 ± 5 %). Remarkably, the decrease in membrane capacitance was even larger than after 4 hours incubation in Gly-Gln.

In a second approach it was tested whether PKC conveys the effects caused by prolonged exposure of rPept1-expressing oocytes to Gly-Gln. For this purpose, experiments with bisindolylmaleimide I (BIM), a highly selective competitive inhibitor of PKC (Ki = 10 nM) were performed.

rPept1-expressing oocytes were simultaneously exposed to either PDBu (1 µM) and BIM (5 µM) for 1 hour or Gly-Gln (5 mM) and BIM (5 µM) for 4 hours. Transport currents and membrane capacitances were measured before and after incubation.

The simultaneous presence of BIM and PDBU completely inhibited the diminishing effects on transport activity and membrane capacitance observed after activation of PKC (Tab. 3.8.1 and Fig. 3.8.1), whereas the presence of BIM in the dipeptide-containing solution failed to block the reduction in transport activity of rPept1: Transport currents

were reduced by about 60 % and the mean membrane capacitance decreased by about 34 %.

To summarize, this data demonstrate that BIM completely reversed the PDBu-induced reduction in transport activity of rPept1 but did not inhibit the dipeptide-induced decrease in transport current and membrane capacitance.

| | transport current [nA] | | membrane capacitance [nF] | |
|---|---|---|---|---|
| | **before** | **after** | **before** | **after** |
| **PDBu** (N/n=2/9) | -401 ± 25 | -198 ± 12** | 340 ± 20 | 145 ± 9** |
| **PDBu + BIM** (N/n=2/11) | -346 ± 33 | -341 ± 34 | 282 ± 5 | 241 ± 9 |
| **Gly-Gln** (N/n=2/9) | -505 ± 49 | -149 ± 17** | 342 ± 6 | 224 ± 3** |
| **Gly-Gln + BIM** (N/n=2/16) | -603 ± 151 | -241 ± 60 | 318 ± 79 | 209 ± 52 |

**Tab. 3.8.1**: Transport currents and membrane capacitances of rPept1-expressing oocytes before and after exposure to 1 µM PDBu for 1 hour, simultaneous exposure to PDBu and BIM for 1 hour, exposure to Gly-Gln for 4 hours and simultaneous incubation in Gly-Gln and BIM for 4 hours
*/**) compared to the data before incubation of the same group

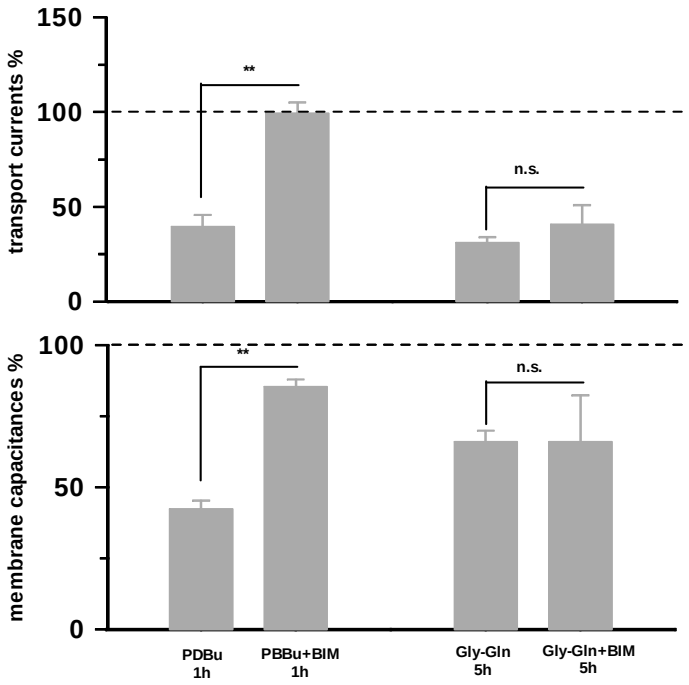


**Fig. 3.8.1:** Transport currents and membrane capacitances of rPept1-expressing oocytes in % after exposure to Gly-Gln, Gly-Gln + BIM, PDBu and PBDu+BIM (100 % indicates the values before incubation)

### 3.8.2 Depolarisation of the cell membrane: comparison between rPept1- and hSGLT1-expressing oocytes

Exposure to substrate evokes depolarisation of the cell membrane and –due to the symport of $H^+$ ions and depending on the buffer capacity of the cytosol– may evoke an acidification. To find out if these changes trigger or contribute to the observed endocytosis of the transporters, differences between the proton-coupled peptide transporter Pept1 and the sodium-dependent glucose transporter SGLT1 were determined. Since both transporters are rheogenic, the depolarisation of the cell membrane can be expected to be similar, but in the case of SGLT1, due to the symport of sodium ions, no intracellular acidification takes place.

For this purpose, first membrane potential, substrate-induced depolarisation and transport currents were measured in rPept1- and human SGLT1-expressing oocytes. The non-hydrolyzed glucose-analogue α-MDG (methyl-α-D-gluco-pyranoside) was used at nearly saturating concentration (5 mM) as substrate for SGLT1.
The average membrane depolarisation of rPept1-expressing oocytes was 61 ± 4 mV, with mean transport currents of -409 ± 44 nA. hSGLT1-expressing oocytes responded with a mean membrane depolarisation of 59 ± 2 mV which was almost identical with that of rPept1-expressing oocytes and exhibited average transport currents of –965 ± 73 nA (Tab. 3.8.2).

| transporter | substrate | Depolarisation of the cell membrane (mV) | transport currents (nA) |
|---|---|---|---|
| **rPept1 (N/n=2/13)** | **5 mM Gly-Gln** | -409 ± 44 | 61 ± 4 |
| **hSGLT1 (N/n=2/11)** | **5 mM α-MDG** | -965 ± 73 | 59 ± 2 |

**Tab. 3.8.2:** Substrate induced depolarisation of the cell membrane and transport currents in the absence and presence of substrate of rPept1- and hSGLT1-expressing oocytes

### 3.8.3 Prolonged exposure of hSGLT1-expressing oocytes to substrate

Depolarisation of the cell membrane in the presence of substrate was similar between rPept1- and hSGLT1-expressing oocytes. If the substrate-induced decrease in transport activity of rPept1 was triggered by the depolarisation of the cell membrane, similar diminishing changes in transport activity should occur when hSGLT1-expressing oocytes are exposed to a substrate-containing solution. To prove this, hSGLT1-expressing oocytes were exposed to a saturating concentration of α-MDG (5 mM) for 5 hours, and transport currents (using 5 mM α-MDG) and membrane capacitances were determined before and after incubation.

In contrast to rPept1, transport currents of hSGLT1 did not decrease but even slightly increased from –518 ± 30 nA before to –630 ± 36 nA after exposure to α-MDG. The mean membrane capacitance of hSGLT1 remained unchanged with mean values of 187 ± 2 nF before and 191 ± 3 nF after incubation (N/n = 3/18). Thus, depolarisation of the cell membrane alone did not trigger the endocytosis of transporters but an other mechanism must have been responsible for the decrease of rPept1 activity after exposure to substrate.

### 3.8.4 Effects of an intracellular accumulation of protons on the transport activity of rPept1

#### 3.8.4.1 Impact of a prolonged exposure to substrate on intracellular pH ($pH_i$) of rPept1-expressing oocytes

The previous experiments showed that exposure to α-MDG did not reduce the transport activity of the sodium-dependent glucose transporter SGLT1, while dipeptides diminished the activity of Pept1. Since exposure of rPept1-expressing oocytes to substrate lead -due to the symport of protons- to an intracellular accumulation of protons, this might exceed the buffer capacity of the oocytes and in turn, acidify the cytosol. Therefore, the next experiments aimed to test whether an intracellular acidification triggered the endocytosis of Pept1 from the oocyte membrane surface.

For this purpose, intracellular pH ($pH_i$) was recorded after exposure of rPept1-expressing oocytes to Gly-Gln (5 mM for 4 hours) and compared to the $pH_i$ of control oocytes (incubated in ND96 solution without substrate) according to the protocol in section 2.3.3.

Control oocytes exhibited an intracellular pH of about 7.70 ± 0.05 (N/n = 2/5), which is in accordance with previous studies (Rodeau et al., 1998; Sasaki et al., 1992). In contrast, the mean cytosolic pH of the dipeptide-treated oocytes (N/n = 2/8) was significantly lowered to 7.18 ± 0.08 ($p < 0.01$) after incubation.

#### 3.8.4.2 Intracellular acidification and its implications on the transport activity of rPept1

The previous experiment indicated that prolonged exposure to substrate, due to the symport of protons, lowered cytosolic pH by about 0.5 units.
The objective of the next series of experiments was to clarify whether lowering the intracellular pH by this extent, but without simultaneous transport of substrate could also decrease transport activity of rPept1 (by decreasing the driving force).

In a previous study (Forster et al., 2000), sodium butyrate was found to evoke a fast and large intracellular acidification in *Xenopus* oocytes expressing the renal $Na^+$-coupled phosphate cotransporter type II. According to this study, a series of experiments was performed with sodium butyrate to acidify the cytosol of rPept1-expressing oocytes in the absence of substrate.

##### 3.8.4.2.1 *Short-term perfusion of rPept1-expressing oocytes with sodium butyrate*

After measuring transport currents in acid-free solution (Fig. 3.8.4.1 mark 1), rPept1-expressing oocytes were perfused with 30 mM sodium butyrate pH 6.2. When intracellular acidification reached its maximum (mark 2) transport currents were measured again. Then, oocytes were washed with ND96 solution until full recovery of $pH_i$ to the value before acid perfusion.

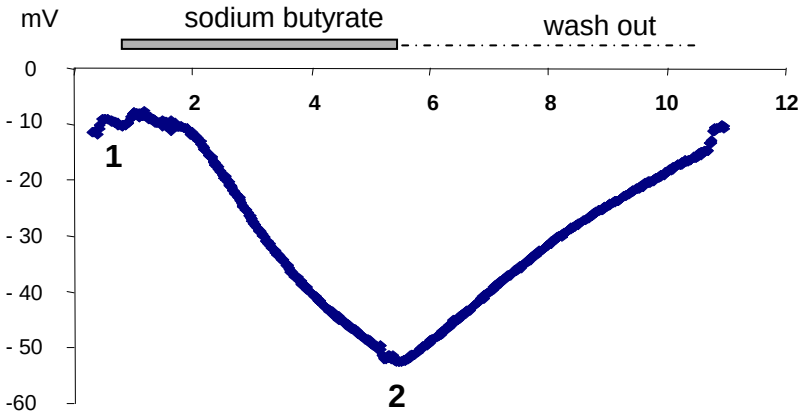


**Fig 3.8.4.1:** $pH_i$ of rPept1-expressing oocytes before, during and after perfusion with 30 mM sodium butyrate pH 6.2

The mean initial transport current of rPept1-expressing oocytes was -320 ± 55 nA with a corresponding $pH_i$ of 7.31 ± 0.03 (1). During perfusion with sodium butyrate, $pH_i$ was lowered to 6.72 ± 0.05 whereas transport currents did not decline but even slightly increased to -369 ± 67 nA (2). After washing out, $pH_i$ rose again to 7.30 ± 0.05. Transport currents increased after recovery to –640 ± 76 nA (N/n = 2/7).

### 3.8.4.2.2 *Long-term exposure of rPept1-expressing oocytes to sodium butyrate*

To investigate the long-term effects of an intracellular acid load on the transport activity of Pept1, oocytes were treated with 10 mM sodium butyrate for 3 hours, oocytes incubated in ND96 solution served as controls.
Transport currents were measured before and after incubation, since oocytes would not have tolerated two $pH_i$ measurements (impaled with a third microelectrode), $pH_i$ was only recorded after incubation period and it was assumed that the initial $pH_i$ of the randomly selected oocytes was nearly identical in the control and experimental groups.

Intracellular pH of the control group was 7.34 ± 0.02 whereas oocytes exposed to sodium butyrate responded with a decline in $pH_i$ of 6.91 ± 0.03. Transport currents in both experimental groups did not decrease but increased after incubation by about 43 % in control oocytes ($p < 0.01$) and by about 28 % in oocytes treated with sodium butyrate ($p < 0.05$ / Tab. 3.8.4.2).

| | transport currents (nA) before | after | $pH_i$ after incubation |
|---|---|---|---|
| **Control** (N/n = 2/7) | -296 ± 44 | -410 ± 47** | 7.34 ± 0.02 |
| **sodium butyrate** (N/n = 2/7) | -292 ± 23 | -378 ± 46* | 6.91 ± 0.03 $^{++}$ |

**Tab. 3.8.4.2:** Transport currents and intracellular pH ($pH_i$) of rPept1-expressing oocytes before and after treatment with 10 mM sodium butyrate or control solution
*/**) compared to the data before incubation of the same group
$^{++}$) comparison of pHi between control group and oocytes treated with sodium butyrate

## 3.9 Co-expression experiments with rPept1, hSGLT1 and mPAT1

The next set of experiments aimed to clarify whether the substrate-induced decrease in transport activity and membrane surface expression is specific for Pept1 or occurs uniformly to other membrane transporters, too. Previous experiments have already indicated that exposure of SGLT1 to α-MDG did not alter its transport parameters. Unclear by now is, whether transporter proteins are regulated differently when co-expressed in the same oocyte. For this purpose, co-expression experiments with rPept1, hSGLT1 and the proton-coupled amino acid transporter PAT1 from mouse (mPAT1) were performed.

### 3.9.1 Prolonged exposure of oocytes co-expressing rPept1 and hSGLT1 to either Gly-Gln and α-MDG

First, the proton-coupled transporter rPept1 and the sodium-coupled transporter hSGLT1 were co-expressed in *Xenopus* oocytes. Transport currents were recorded before and after exposure to substrate (5 mM Gly-Gln for rPept1 and 5 mM α-MDG for hSGLT1).

The findings in Tab 3.9 and Fig 3.9.1 indicate that exposure of oocytes, co-expressing rPept1 and hSGLT1 for 4 hours to Gly-Gln reduced only the dipeptide-induced currents (to 40 ± 2 %; $p < 0.01$ compared to the data before incubation), while transport currents evoked by α-MDG did not change significantly. The average membrane capacitance decreased only moderately from 231 ± 3 to 205 ± 6 nF. When rPept1 and hSGLT1 co-expressing oocytes were treated for the same time period with α-MDG instead of Gly-Gln,

transport currents of rPept1 increased to 151 ± 6 % ($p < 0.01$; compared to the data before incubation), whereas the transport activity and membrane capacitance of hSGLT1 were not altered.

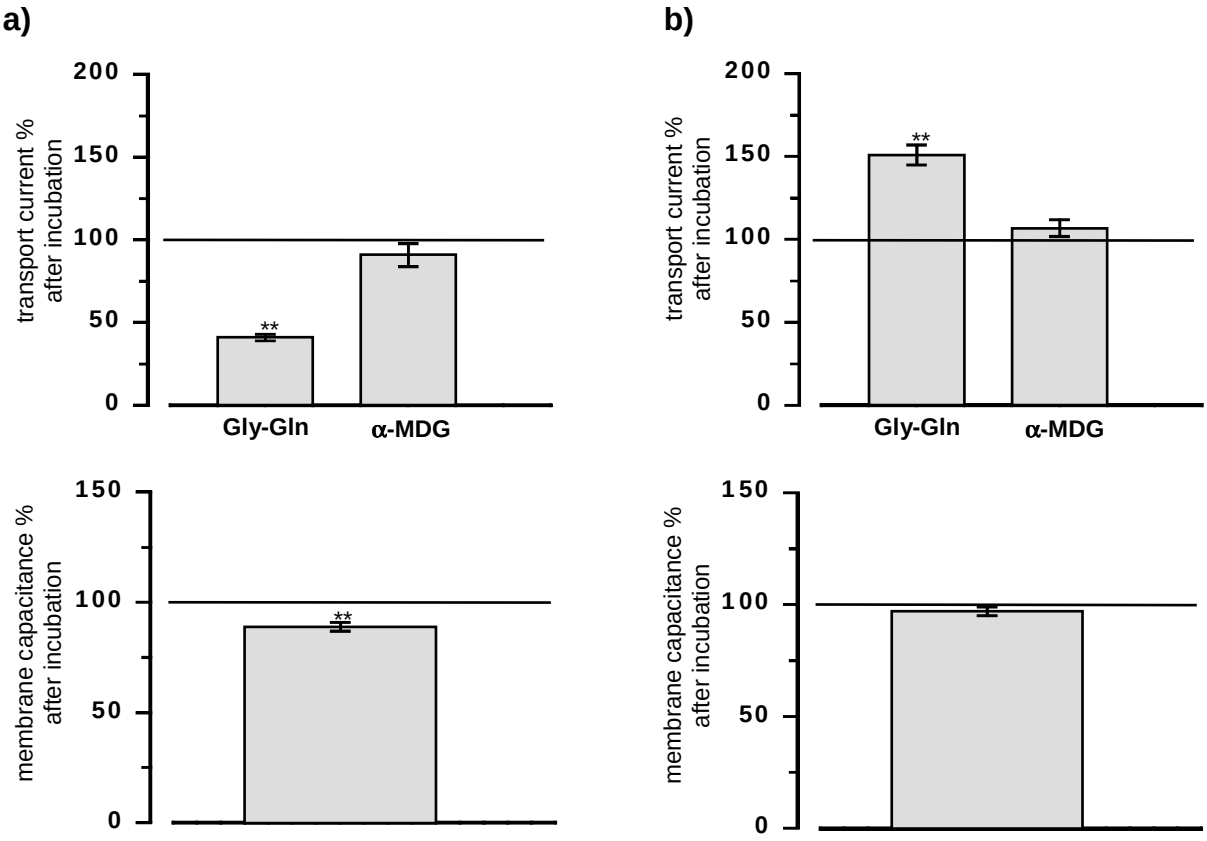


**Fig. 3.9.1:** Peptide- and glucose-induced transport currents and membrane capacitances after incubation of rPept1- and hSGLT1-coexpressed oocytes for 4 hours in **a)** 5 mM Gly-Gln and **b)** 5 mM α-MDG (100 % represents the values before incubation)
**) compared to the data before incubation

### 3.9.2 Prolonged exposure of oocytes co-expressing rPept1 and mPAT1 to either cefadroxil or glycine

Similar co-expression experiments were performed with the two proton-coupled transporters rPept1 and mPAT1. These oocytes were exposed to a nearly saturating concentration of cefadroxil (20 mM) or glycine (20 mM) latter is a good substrate for mPAT1. Cefadroxil instead of the dipeptide Gly-Gln was used as incubation substrate since intracellular hydrolysis of Gly-Gln would have increased cytosolic amino acid concentration and thus could have effected the function of mPAT1. Transport currents and

membrane capacitances were determined with 5 mM Gly-Gln (rPept1) or 20 mM glycine (mPAT1) before and after substrate exposure.

Treatment with cefadroxil reduced transport currents of both transporters to a similar degree (Fig 3.9.2 and Tab. 3.9): the dipeptide-induced transport currents decreased to 59 ± 7 %, the glycine-induced currents to 63 ± 8 % ($p < 0.01$; both compared to the data before incubation). The membrane capacitance declined from 247 ± 10 nF to 214 ± 10 nF ($p < 0.01$).

Almost identical effects on transport activity and membrane capacitance of rPept1 and mPAT1 were observed after exposure to glycine. Average transport currents of both transports were reduced by about 40 %. Membrane capacitance declined from 238 ± 9 nF to 217 ± 6 nF ($p < 0.01$).

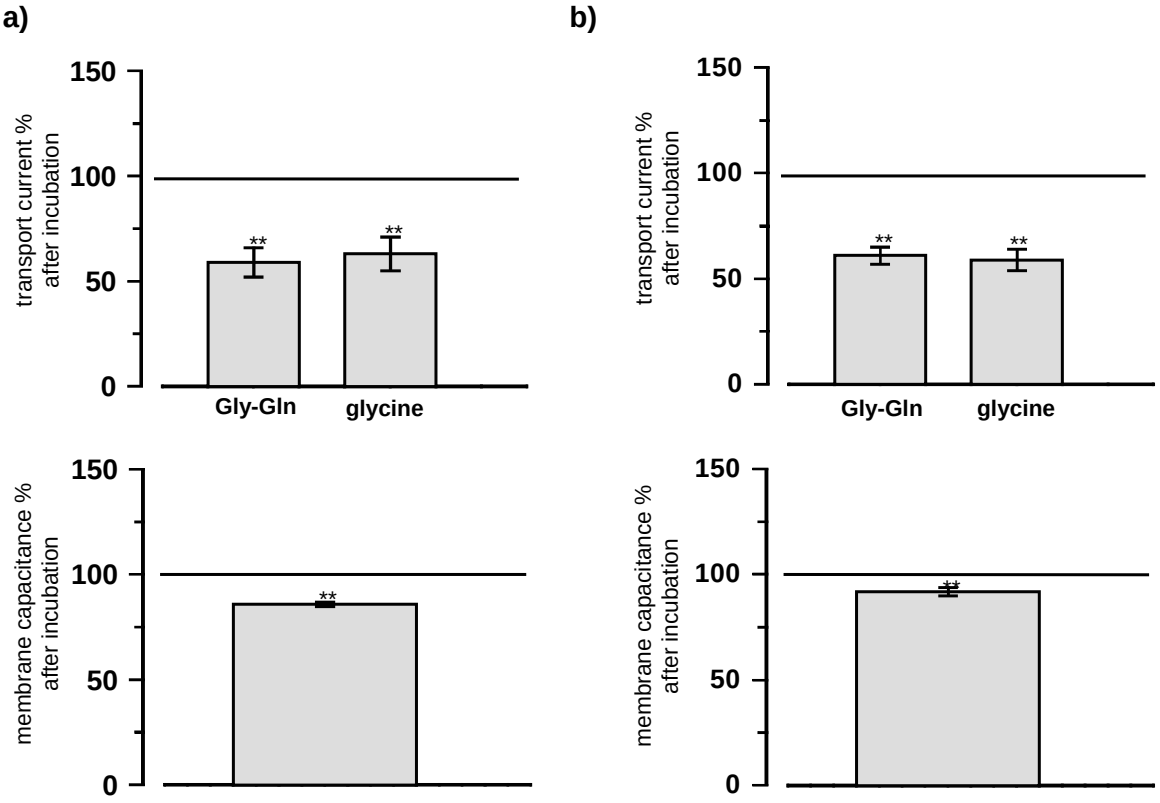


**Fig. 3.9.2:** Peptide- and glycine-induced transport currents and membrane capacitances in % after incubation of rPept1- and mPAT1-coexpressing oocytes for 4 hours in **a)** 20 mM cefadroxil and **b)** 20 mM glycine (100 % represents the values before incubation)
**) compared to the data before incubation

### 3.9.3 Prolonged exposure of oocytes co-expressing mPAT1 and hSGLT1 to either glycine or α-MDG

In a third approach hSGLT1 and mPAT1 were co-expressed in oocytes and exposed to either 5 mM α-MDG or 20 mM glycine for 4 hours. Transport currents and membrane capacitances of mPAT1 were determined with 20 mM glycine, transport parameters of hSGLT1 with 5 mM α-MDG before and after substrate treatment.

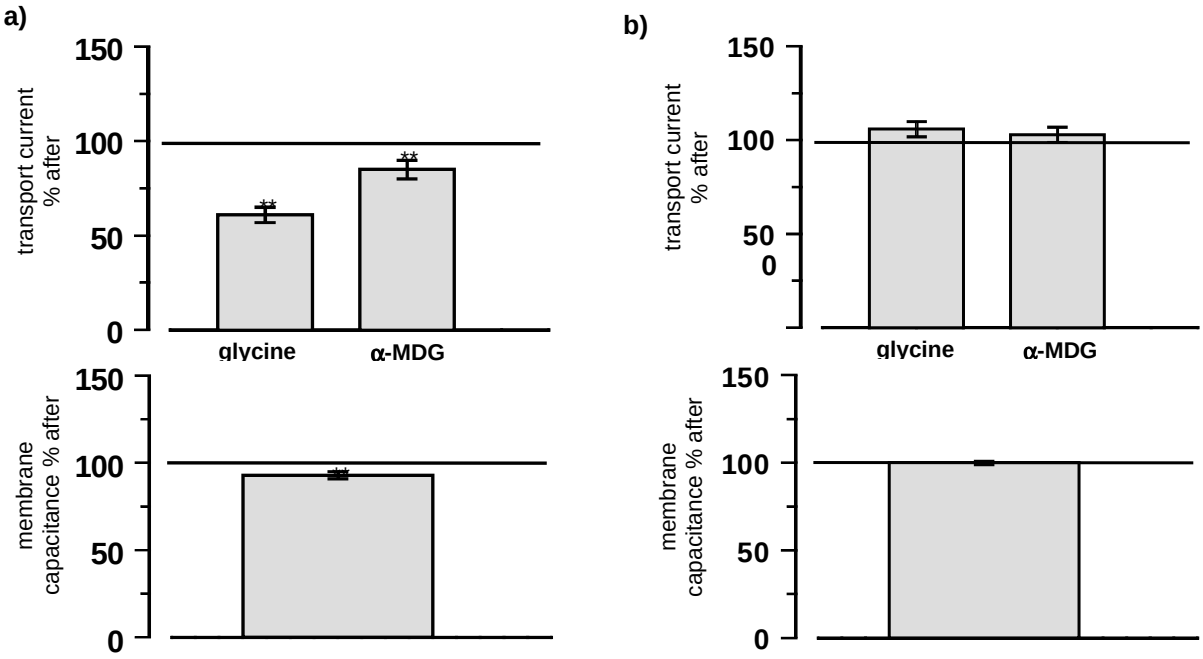


**Fig 3.9.3:** Co-expression of mPAT1 and hSGLT1: The figures show the glycine- and α -MDG induced transport currents and membrane capacitances after **a)** exposure to 20 mM cefadroxil and **b)** exposure to 5 mM α-MDG for 4 hours (100 % represents the data before incubation)
**) compared to the data before incubation

Exposure to glycine evoked a reduction in transport activity of hSGLT1 by about 15 %, which was significant, but only modest as compared to the decrease in mPAT1-related currents of about 40 %. Membrane capacitance remained almost unchanged after exposure to both substrates.

Transport currents as well as membrane capacitances of both mPAT1 and hSGLT1 did not alter after exposure to α-MDG. All results are presented in Fig 3.9.3 and Tab. 3.9.

| co-expressed transporters | rPept1 + mPAT1 (1) | (2) | rPept1 + hSGLT1 (1) | (2) | mPAT1 + hSGLT1 (1) | (2) |
|---|---|---|---|---|---|---|
| **incubated in** | **cefadroxil (5 mM)** | **glycine (20 mM)** | **Gly-Gln (5 mM)** | **α–MDG (5 mM)** | **glycine (20 mM)** | **α–MDG (5 mM)** |
| **N/n** | **2/13** | **3/14** | **3/26** | **2/12** | **4/23** | **4/17** |
| **current 1 (nA)** | | | | | | |
| before | 232 ± 37 | 352 ± 38 | 294 ± 16 | 284 ± 10 | 320 ± 17 | 322 ± 19 |
| after | 152 ± 29 | 215 ± 18 | 119 ± 9 | 426 ± 19 | 190 ± 13 | 334 ± 17 |
| after in % | 59 ± 7** | 61 ± 4** | 41 ± 2** | 151 ± 6** | 61 ± 4** | 106 ± 4 |
| **current 2 (nA)** | | | | | | |
| before | 200 ± 35 | 340 ± 28 | 237 ± 19 | 353 ± 15 | 348 ± 31 | 384 ± 41 |
| after | 135 ± 27 | 222 ± 21 | 191 ± 9 | 373 ± 17 | 301 ± 33 | 408 ± 56 |
| after in % | 63 ± 8** | 59 ± 5** | 91 ± 7 | 107 ± 5 | 85 ± 5** | 103 ± 4 |
| **Capacitance (nF)** | | | | | | |
| before | 214 ± 10 | 238 ± 9 | 231 ± 3 | 246 ± 4 | 184 ± 9 | 186 ± 12 |
| after | 247 ± 10 | 217 ± 6 | 205 ± 6 | 239 ± 5 | 168 ± 6 | 186 ±11 |
| after in % | 86 ± 1** | 92 ± 2** | 89 ± 2** | 97 ± 2 | 93 ± 2** | 100 ± 1 |

**Tab. 3.9:** Exposure of co-expressed transporters (rPept1 and mPAT1, rPept1 and hSGLT1, mPAT1 and hSGLT1) to different substrates for 4 hours. Transport currents and membrane capacitances were measured before and after incubation: Pept1-mediated currents with 5 mM Gly-Gln, hSGLT1-mediated currents with 5 mM α-MDG and mPAT1-induced currents with 20 mM glycine.
**) compared to the data before incubation

# 4 RESULTS: CELL CULTURE EXPERIMENTS

## 4.1 Prolonged exposure of Caco-2 cells to dipeptide containing solutions

Previous studies in the human carcinoma cell line Caco-2 indicated that prolonged exposure to dipeptide-containing solutions (Gly-Sar or Gly-Gln) resulted in an increase in Pept1 activity (Thamotaran et al., 1997; Walker et al., 1998). In contrast, in *X.* oocytes, transport activity of Pept1 did not increase but declined after exposure to Gly-Gln. This effect was investigated after a 4 hours incubation period, whereas the stimulating effects on Pept1 activity in Caco-2 cells were observed after 24 h, respectively 3 days. In the next set of experiments incubation period of Caco-2 cells in dipeptide-containing solutions was shortened to 8 hours to observe potential short-term effects on Pept1 activity. As incubation substrates served the dipeptides Gly-Sar, Gly-Gln, Gly-Gly or Gly-Leu (each 5 mM).

Prolonged exposure of Caco-2 cells for 8 hours to Gly-Sar, Gly-Gln or Gly-Gly significantly increased $^{14}$[C]Gly-Sar uptake rate about 71 ± 5.7 %, 34 ± 6.8 % and 46 ± 21.1 % in contrast to the control group. $^{14}$[C]Gly-Sar uptake rate after exposure to the dipeptide Gly-Leu (Fig 4.1 a) was equal to that of the control cells.

Inhibition of proteinase activity with bestatin (10 µM) during exposure of Caco-2 cells to the dipeptides Gly-Gln or Gly-Gly even enlarged the stimulating effect on $^{14}$[C]Gly-Sar uptake rate (Fig 4.1 b). However, application of bestatin to the Gly-Sar or Gly-Leu containing incubation medium had no additional positive effect on $^{14}$[C]Gly-Sar uptake rate. The data in Tab. 4.1 indicate that the increase in $^{14}$[C]Gly-Sar uptake rate after exposure to dipeptide-containing solutions is paralleled with an augmentation of the protein levels of the cells.

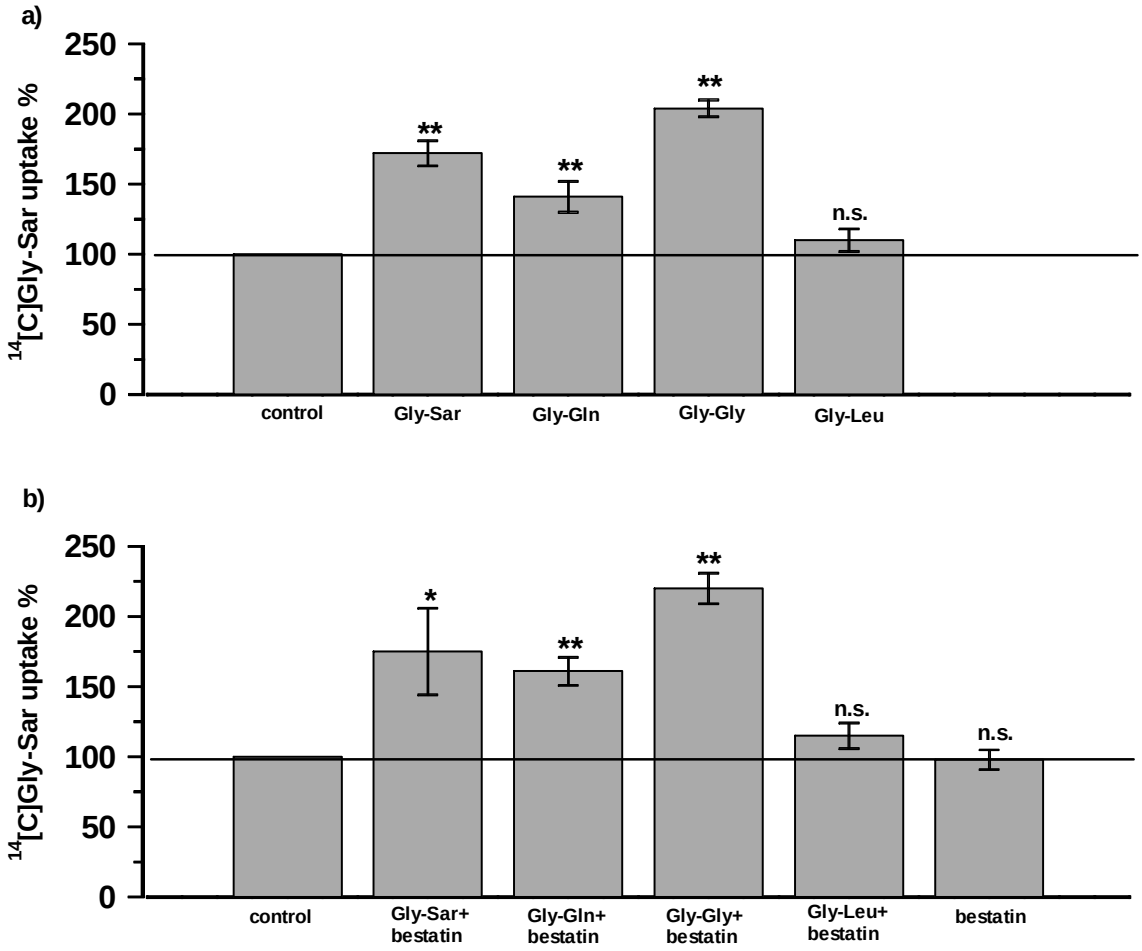


**Fig. 4.1:** Exposure of Caco-2 cells to saturating dipeptide solutions containing either Gly-Gln, Gly-Sar, Gly-Leu or Gly-Gly (each 5 mM) for 8 hours **a)** in the absence or **b)** in the presence of the proteinase inhibitor bestatin (10 µM)
*/** compared to the control group

| | Uptake pmol/cm²*10min | Uptake pmol/mg protein | dipeptide/control pmol/cm²*10min | dipeptide/control pmol/mg protein |
|---|---|---|---|---|
| **Gly-Sar (N/n=5/12)**<br>**Control (N/n=5/11)** | 42.3 ± 5.7<br>24.6 ± 3.2 | 484 ± 32<br>292 ± 26 | 1.72 ± 0.09** | 1.68 ± 0.09** |
| **Gly-Sar +bestatin (N/n=2/4)**<br>**Control (N/n=2/6)** | 49.5 ± 8.9<br>35.6 ± 6.1 | ---- | 1.75 ± 0.31* | ---- |
| **Gly-Gln (N/n=5/14)**<br>**Control (N/n=5/13)** | 39.4 ± 3.0<br>28.2 ± 1.6 | 411 ± 17<br>312 ± 17 | 1.41 ± 0.11* | 1.36 ± 0.11* |
| **Gly-Gln + bestatin (N/n=6/114)**<br>**Control (N/n=6/14)** | 43.0 ± 5.0<br>26.5 ± 2.2 | 490 ± 30<br>301 ± 19 | 1.61 ± 0.10** | 1.61 ± 0.11**/$^{+}$ |
| **Gly-Gly (N/n=4/12)**<br>**Control (N/n=4/14)** | 40.3 ± 5.7<br>24.5 ± 3.6 | 417 ± 305<br>268 ± 24 | 2.04 ± 0.06** | 2.00 ± 0.08** |
| **Gly-Gly + bestatin (N/n=4/11)**<br>**Control (N/n=4/14)** | 48.5 ± 4.4<br>24.5 ± 3.6 | 517 ± 33<br>268 ± 24 | 2.20 ± 0.11** | 2.21 ± 0.08**/$^{+}$ |
| **Gly-Leu (N/n=2/4)**<br>**Control (N/n=2/6)** | 32.7 ± 7.7<br>35.6 ± 7.9 | ---- | 1.10 ± 0.08 | ---- |
| **Gly-Leu +bestatin (N/n=2/4)**<br>**Control (N/n=2/6)** | 34.2 ± 6.5<br>35.6 ± 7.9 | ---- | 1.15 ± 0.09 | ---- |

**Tab. 4.1:** $^{14}$[C]Gly-Sar uptake rates after pre-exposure of Caco-2 cells for 8 hours in saturating concentrations of Gly-Sar, Gly-Gln or Gly-Gly in the absence or presence of 10 µM bestatin
*/** compared to the control group
$^{+}$) compared to the group without bestatin

## 4.2 Amino acid analysis of the cytosol of Caco-2 cells after exposure to dipeptides

The previous results indicated that prolonged exposure of Caco-2 cells to the dipeptides Gly-Sar, Gly-Gln or Gly-Gly resulted in a markedly increase in $^{14}$[C]Gly-Sar uptake. In the case of Gly-Gln or Gly-Gly, this effect was even enlarged by inhibition of proteinase activity during incubation period. This suggests that high amounts of intracellular dipeptides may trigger the increase in Pept1 activity.

In the following, an amino acid analysis of the cytosol of Caco-2 cells after exposure to dipeptides was performed to observe whether bestatin indeed blocked hydrolysation of cytosolic dipeptides.

In control cells no significant amounts of amino acids or peptides were detected. In the absence of bestatin, the dipeptides Gly-Gln and Gly-Gly were hydrolysed for the most part, whereas no amounts of Gly-Leu were found. Relative huge amounts of Gly-Sar -a dipeptide which is very unlike to be hydrolysed- were even found in the absence of bestatin. When bestatin was added to the dipeptide containing medium, hydrolysis of the dipeptides Gly-Gln and Gly-Gly was mainly blocked. However, inhibition of proteinases hardly prevented hydrolysis of Gly-Leu (Tab. 4.2).

| | **glutamine (µmol/l)** | **glycine (µmol/l)** | **leucine (µmol/l)** | **Gly-Gln (µmol/l)** | **Gly-Sar (µmol/l)** | **Gly-Gly (µmol/l)** | **Gly-Leu (µmol/l)** |
|---|---|---|---|---|---|---|---|
| **Gly-Sar** (N/n=2/4) | | 43.7 ± 0.6 | | | 18.5 ± 0.7 | | |
| **Gly-Gln** (N/n=2/4) | 50.5 ± 1.5 | 45.5 ± 1.2 | | 0.0 ± 0 | | | |
| **Gly-Gln + bestatin** (N/n=2/4) | 0.0 ± 0 | 18.6 ± 2.8 | | 25.7 ± 3.5 | | | |
| **Gly-Gly** (N/n=2/4) | | 62.7 ± 3.7 | | | | 2.2 ± 0.1 | |
| **Gly-Gly + bestatin** (N/n=2/4) | | 20.8 ± 2.4 | | | | 29.8 ± 3.0 | |
| **Gly-Leu** (N/n=2/4) | | 46.6 ± 2.3 | 44.9 ± 1.8 | | | | 0.0 ± 0 |
| **Gly-Leu + bestatin** (N/n=2/4) | | 31.0 ± 2.1 | 19.7 ± 1.6 | | | | 4.8 ± 0.2 |
| **Control** (N/n=2/4) | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 | 0.0 ± 0 |

**Tab. 4.2:** Amino acid analysis of the cytosol of Caco-2 cells after 8 hours exposure to various dipeptides (± bestatin)

## 4.3 Effect of cytochalasin D on $^{14}$[C]Gly-Sar uptake rate in Caco-2 cells

Increased transport activity of Pept1 after exposure to dipeptides might have been the result of a redistribution of transporter proteins from intracellular compartments to the membrane surface. To test this possibility, Caco-2 cells were pre-exposed to the cytoskeleton modifier cytochalasin D (CD) in the absence or presence of dipeptides for 8 h.

$^{14}$[C]Gly-Sar uptake rates of cells treated with only 2 µM CD or control solution were not significant different (Tab. 4.3). The presence of CD in the Gly-Sar or Gly-Gln containing incubation medium resulted in a slight, but not significant decrease in $^{14}$[C]Gly-Sar uptake rate by about 10 % and 8 %, respectively (11 % in the presence of bestatin) as compared to the cells pre-exposed to dipeptides without CD.

| | **pmol/mg protein** | **dipeptide+CD/dipeptide** |
|---|---|---|
| **CD (N/n=2/6)** | 179 ± 5 (n.s.) | |
| **Control (N/n=2/6)** | 215 ± 19 | |
| **Gly-Sar (N/n = 3/9)** | 540 ± 14** | |
| **Gly-Sar + CD (N/n = 3/9)** | 488 ± 25** | 0.90 (n.s.) |
| **Control (N/n = 3/8)** | 330 ± 25 | |
| **Gly-Gln (N/n=4/12)** | 409 ± 19 (n.s.) | |
| **Control (N/n=4/11)** | 323 ± 18 | |
| **Gly-Gln + CD (N/n=2/5)** | 345 ± 29 (n.s) | 0.82 (n.s.) |
| **Control (N/n=2/5)** | 305 ± 4 | |
| **Gly-Gln + bestatin (N/n=4/11)** | 539 ± 19** | |
| **Gly-Gln + bestatin + CD (N/n=4/12)** | 486 ± 25** | 0.89 (n.s.) |
| **Control (N/n=4/14)** | 323 ± 18 | |

**Tab. 4.3:** Simultaneous exposure of Caco-2 cells to cytochalasin D (CD; 2 µM) and Gly-Sar or Gly-Gln (with/without bestatin) for 8 hours. Uptake rate was measured with $^{14}$[C]Gly-Sar
**) compared to the control group

## 4.4 Effects of brefeldin A on $^{14}$[C]Gly-Sar uptake rate in Caco-2 cells

To test the possibility that the increase in $^{14}$[C]Gly-Sar uptake rate after exposure to dipeptide-containing solutions was due to an insertion of newly synthesised proteins into the cell membrane, Caco-2 cells were pre-exposed to brefeldin A (BFA).
BFA disrupts the Golgi complex, which in turn inhibits trafficking of vesicles to the cell membrane and as a consequence protein secretion and membrane insertion is blocked. (Liu et al., 2001; Weber et al., 2001, Maroto et. al, 2001).

Treatment of Caco-2 cells for 8 hours with 50 µM BFA caused a large decrease in $^{14}$[C]Gly-Sar uptake rate as compared to the control group (Tab. 4.4). This effect of BFA was about equal in the presence as well as in the absence of dipeptides (Fig. 4.4 a/b, Tab. 4.4). As seen before, pre-exposure of Caco-2 cells to the dipeptides Gly-Sar or Gly-Gln (each 5 mM) in the presence or absence of bestatin resulted in a marked increase in $^{14}$[C]Gly-Sar uptake rate. This stimulating effect was completely reversed by simultaneous application of BFA to the dipeptide-containing incubation medium (Fig.4.4 a/b, Tab. 4.4), suggesting that prolonged exposure of Caco-2 cells to dipeptides triggered synthesis of peptide transporter proteins.

| | pmol/mg protein | BFA / control | dipeptide+BFA / dipeptide | Dipeptide/ control | dipeptide+BFA /control |
|---|---|---|---|---|---|
| | | **BFA effect** | | **dipeptide effect** | |
| | | **- dipeptide** | **+ dipeptide** | **- BFA** | **+ BFA** |
| **Control N/n=4/11** | 321 ± 31 | | | | |
| **BFA N/n=4/12** | 185 ± 11 | 0.58 ± 0.04** | | | |
| **Gly-Sar N/n=3/9** | 540 ± 26 | | 0.48 ± 0.02** | 1.69 ± 0.14** | 0.81 ± 0.06$^{++}$ |
| **Gly-Gln N/n=4/12** | 409 ± 35 | | 0.62 ± 0.03** | 1.32 ± 0,15* | 0.99 ± 0.11$^{++}$ |
| **Gly-Gln+ bestatin N/n=4/11** | 546 ± 36 | | 0.57 ± 0.03** | 1.73 ± 0.13** | 0.87 ± 0.07$^{++}$ |

**Tab. 4.4.:** Effect of 50 µM brefeldin A on $^{14}$[C]Gly-Sar uptake rate after simultaneous incubation in Gly-Sar or Gly-Gln (in the absence or presence of bestatin)
*/** p<0.01 / p<0.001
$^{++}$p<0.001: comparison of the dipeptide effect without BFA (- BFA) and with BFA (+ BFA)

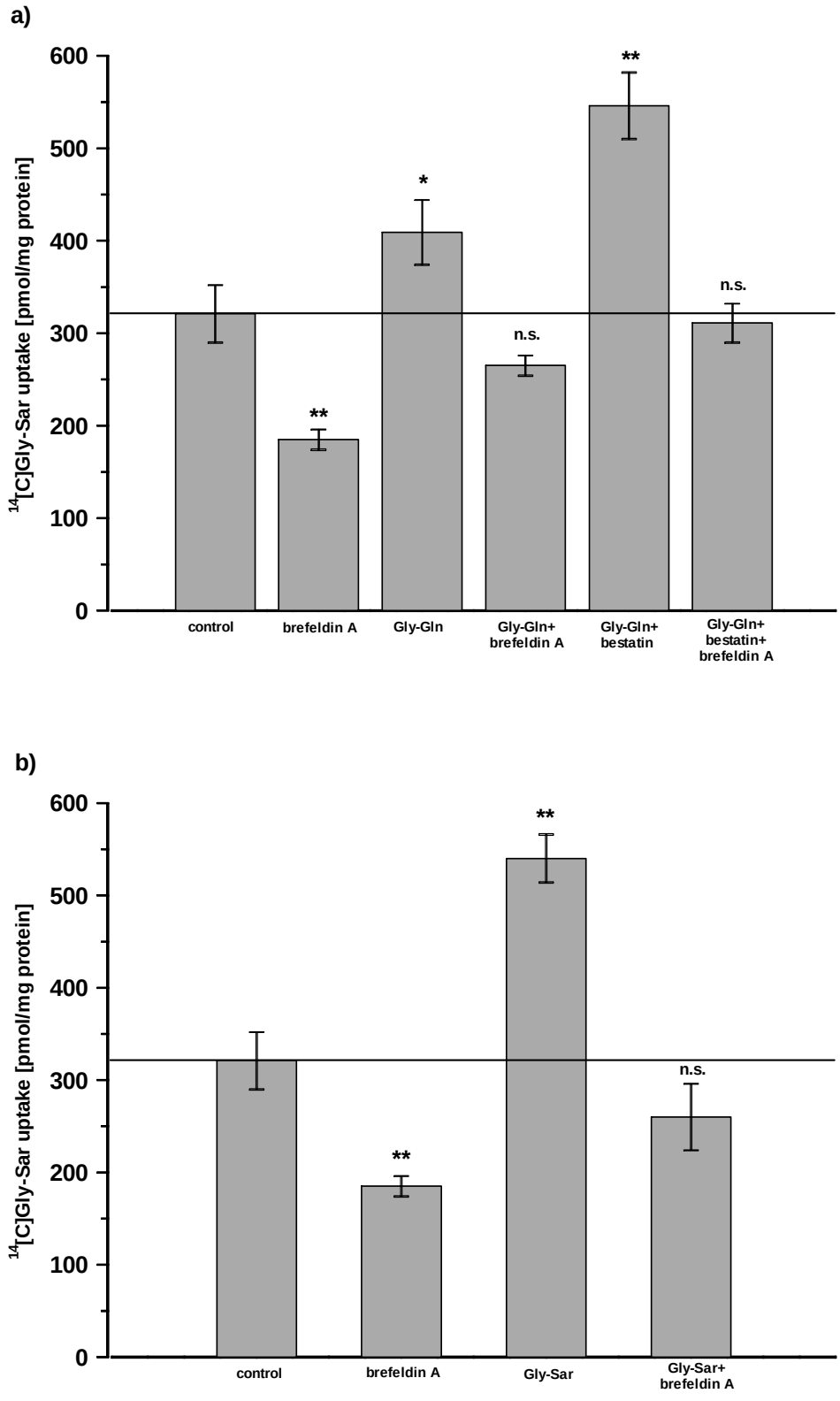


**Fig 4.4:** Effect of 50 µM brefeldin A on $^{14}$[C]Gly-Sar uptake rate after simultaneous pre-exposure to **a)** Gly-Gln in the presence or absence of bestatin and **b)** Gly-Sar
*/** ($p<0.01$ / $p<0.001$) compared to the control group

## 4.5 Pre-exposure of Caco-2 cells to various beta-lactam antibiotics for 8 hours

Studies with *X.* oocytes showed that prolonged exposure to the beta-lactam antibiotic cefadroxil reduced transport activity of rPept1 markedly. The following experiments aimed to observe the effects of various beta-lactam antibiotics on $^{14}$[C]Gly-Sar uptake rate in Caco-2 cells.

For this purpose, Caco-2 cells were for 8 hours pre-exposed to the beta-lactam antibiotics cephalexin, cephradine, captopril or amoxicillin each in a concentration of 5 mM.

As the data in Tab. 4.5 and Fig. 4.5.1 indicate, pre-exposure of Caco-2 cells to cephalexin did not alter $^{14}$[C]Gly-Sar uptake rate in Caco-2 cells as compared to the control group. $^{14}$[C]Gly-Sar uptake rate in Caco-2 cells treated with either cephradine, captopril or amoxicillin was only slightly reduced. However, pre-exposure of Caco-2 cells to cefradroxil decreased $^{14}$[C]Gly-Sar uptake rate significantly by about 35 % as compared to the control group. This effect could have been caused by a reduced trafficking rate of Pept1 proteins or by apoptosis processes. Optically, no differences between cefadroxil-treated and control cells were observed. However, when in further experiments the protein levels in control and cefadroxil-exposed cells were measured and the results were calculated per mg protein, the differences to the control group were no longer significant (Fig. 4.5.2). This suggests that prolonged exposure to high concentrations of cefradroxil may cause apoptosis in Caco-2 cells.

| | **pmol/10 min * cm²** |
|---|---|
| **control** | 31.4 ± 3.1 |
| **cefadroxil** | 17.9 ± 0.2** |
| **cephalexin** | 27.7 ± 0.4 (n.s) |
| **cephradine** | 26.9 ± 0.6* |
| **captopril** | 24.3 ± 2.4* |
| **amoxicillin** | 24.4 ± 2.9* |

**Tab. 4.5:** $^{14}$[C]Gly-Sar uptake rate (pmol/10 min*10cm²) after pre-exposure of Caco-2 cells for 8 hours in various beta-lactam-antibiotics. (N/n=2/6)
*/** compared to the control group

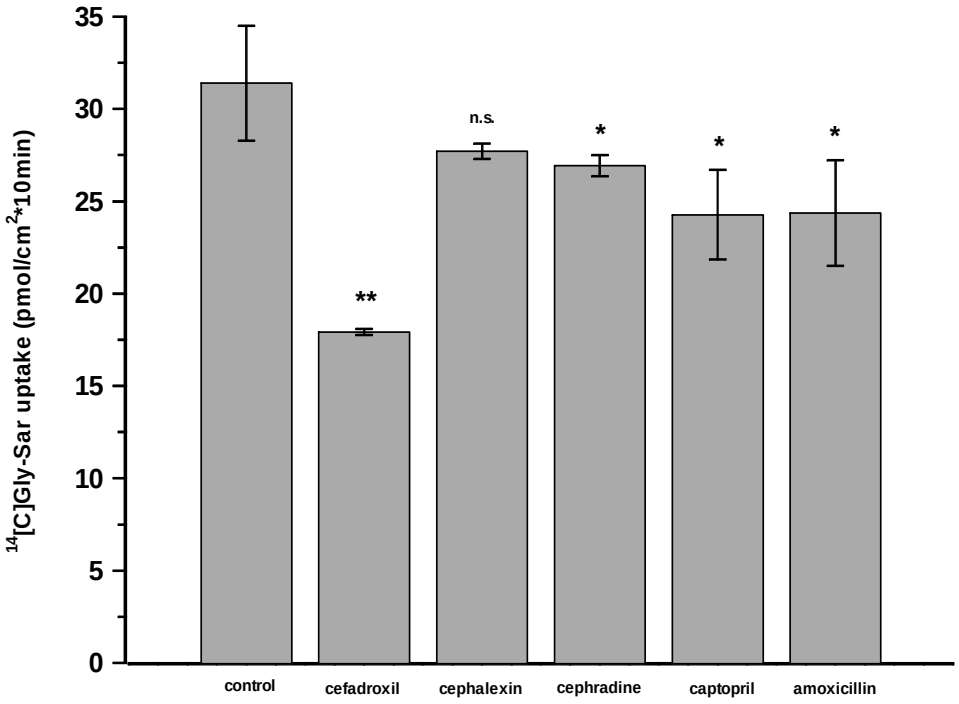


**Fig 4.5.1:** Rate of $^{14}$[C]Gly-Sar uptake (pmol/cm$^2$*10 min) after 8 hours pre-exposure of Caco-2 cells to the beta-lactam antibiotics cefadroxil, cephalexin, cephradine, captopril or amoxicillin (each 5 mM). N/n = 2/6
*/** compared to the control group

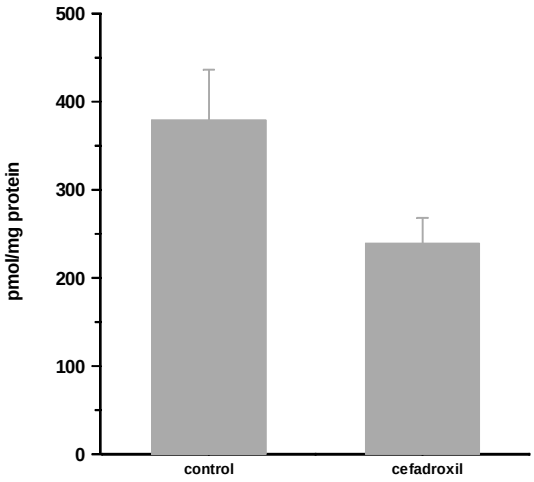


**Fig. 4.5.2:** Rate of $^{14}$[C]Gly-Sar uptake (pmol/mg protein) after 8 hours pre-exposure of Caco-2 cells to 5 mM cefradroxil or control solution (N/n = 4/12). Differences between both groups are not significant

# 5 DISCUSSION

The aim of the present work was to investigate the fate of rPept1 transporter proteins after synthesis in *X.* oocytes by means of electrophysiological and immunofluorescence techniques: From the incorporation of peptide transporter into the oocyte membrane surface to changes in surface expresssion due to endocytotic processes in response to different stimuli such as dipeptides, beta-lactam antibiotics, inhibitors. The role of the cytoskeleton and the impact of protein kinase C in this process were observed. Besides, possible effects of the membrane potential, substrate and proton gradient on the transport activity of rPept1 were examined. Furthermore, differences between regulation processes in *X.* oocytes and Caco-2 cells were identified.

## 5.1 Membrane area changes in *X.* oocytes

Native *X.* oocytes possess average membrane capacitances in the range between 158 nF and 236 nF (Zhu et al., 1997; Forster et al., 1999; Awayda et al., 2000; Valentin et al., 2000; Segal et al., 2002). The mean membrane capacitance of non-injected and water-injected oocytes was about 220 nF. Since the specific capacitance of several lipid bilayers has been found to be about 1 µF/cm$^2$, this value is far more than what could be expected for a smooth globe having the diameter of a typical oocyte. Measurements of the oocyte diameter indicated a surface amplification factor of about six times over a smooth sphere with a diameter of 1.1 mm. The expression of rPept1 in the cell membrane resulted in peptide-induced currents of about 400 to 500 nA paralleled by an increase in membrane capacitance to about 300 nF, thus resulting in a membrane surface amplification factor of about eight to nine times. Moreover, there was a positive correlation between peptide-induced transport currents and membrane capacitances in rPept1-expressing oocytes, suggesting that the insertion of peptide transporters into the membrane surface was accompanied by an increase in membrane surface complexity of the oocyte.

In previous studies, it has already been reported that heterologous expression of transporter or channel proteins was paralleled with a rise in membrane capacitance and surface area. Weber et al. (2001) reported that in oocytes increased expression of the human cystic fibrosis transmembrane conductance regulator (CFTR) resulted in an augmentation of membrane capacitances (from 190 to about 270 nF) in parallel with an increase in chloride currents. The insertion of rabbit SGLT1 transporter proteins into the oocyte membrane was accompanied by a rise in membrane capacitance from about 304 to 594 nF (Hirsch et al., 1996). A decline in membrane capacitance due to reduced

surface expression levels were observed in the case of $Na^+/K^+$-ATPase in oocytes. Electron micrograph analysis indicated changes in the morphology of the oocyte surface after endocytosis of pump proteins: microvilli were reduced in length and number and irregularly oriented (Vasilets et al., 1990). Similar changes in membrane capacitance due to endo- and exocytotic processes were observed in the case of the type II sodium-coupled inorganic phosphate cotransporter (Forster et al., 1999) and the epithelial $Na^+$-channel from rat rENaC (Segal et al. 2002). In contrast, no changes in membrane capacitance due to altered expression levels were observed in the case of mPAT1 (Foltz et al., 2005), the human dopamine transporter hDAT (Zhu et al., 1997) and human SGLT1 (Valentin et al., 2000).

The increase in membrane capacitance in rabbit SGLT1 was equivalent to a net insertion of about 1300 vesicles per second over a period of 6 days into the plasma membrane. Assuming a vesicle diameter of 120 nm, 200 transporter proteins per fused vesicles were calculated. However, since microscopic analysis revealed only 20 SGLT1 proteins per vesicle thus, exocytosis rate of SGLT1 must have been even much higher (Hirsch et al. 1996). For rPept1 a number of about 74 proteins per vesicle was calculated which is similar to the above values or the data determined for glycine and glutamate transporters (Aubrey et al., 2005).

## 5.2 The role of the cytoskeleton in the substrate-induced downregulation of rPept1 activity in *X.* oocytes

In *X.* oocytes, the substrate-induced downregulation of rPept1 activity was almost completely blocked by applying the cytoskeleton-modifier cytochalasin D to the dipeptide-containing medium, thus indicating, that redistribution of transporter proteins from the membrane surface was prevented by destroying the normal function of the cytoskeleton.

A possible explanation for the fact that CD failed to fully inhibit the decrease in transport activity is that CD was added to the dipeptide-containing solution only during the last three hours, since a longer lasting incubation period with CD usually destroyed the oocytes.

In previous studies, evidence was provided that endo- and exocytotic processes of transporter or channel proteins require an intact protein trafficking machinery. Observations in *X.* oocytes with the human cystic fibrosis transmembrane conductance regulator (CFTR) showed that treatment of oocytes with nocodazole, an agent responsible for depolymerization of tubulin monomers, blocked the endocytotic withdrawal of CFTR proteins from the plasma membrane (Weber et al., 2000). A functional intact cytoskeleton

also plays an important role in trafficking and localisation of the $Na^+/H^+$ exchanger NHE3 (Cha et al., 2004), the renal betaine/GABA transporter BGT1 (Basham et al., 2001) and the voltage-dependent potassium channel Kv4.2 (Wang et al., 2004) as shown by the inhibition by latrunculin B (0.05 µM), cycloheximide (20 µM) and cytochalasin D (5 µM), respectively.

## 5.3 Regulation of heterologously expressed transporters by PKC in *X.* oocytes

Former studies have shown that protein kinase C plays an important role in the regulation of the abundance of several transporter proteins in the plasma membrane of *X.* oocytes, but also of other cell types (see below). The present investigations showed that activation of PKC resulted in a marked decline in transport currents (by about 50 %) and membrane capacitance of Pept1 (by about 55 %). This PKC induced reduction in transport activity was to the greatest part reversed by the simultaneous application of the PKC-activator PDBu and the cytoskeleton-modifier cytochalasin D, suggesting that stimulation of PKC triggered an endocytosis of peptide transporters from the membrane surface. The PKC-induced decline in transport activity of rPept1 was blocked by inhibition of PKC with BIM. However, BIM failed to inhibit the decrease in transport currents and membrane capacitance observed after exposure of oocytes to Gly-Gln, suggesting, that PKC is not involved in the substrate-induced downregulation in rPept1 activity.
Noticeable, the decrease in membrane capacitance after 1 hour incubation in PDBu was even larger than after 4 hours exposure to Gly-Gln, which probably was caused by an unspecific intracellular retrieval of not only peptide transporters but also other membrane proteins.

PKC has been found to be involved in trafficking processes of a variety of heterologously expressed transporters in *X.* oocytes. Studies by Hirsch et al. (1996) demonstrated that PKC regulates the activity of SGLT1 by endo- and exocytosis processes. However, the response to activation of PKC depends on the transporter in question: while maximum transport rate declined for rabbit and rat SGLT1, transport activity of human SGLT1 increased. Studies with the $Na^+/K^+$-ATPase showed that activation of PKC induced a reduction of the number of pump molecules in the membrane surface as well as pump currents. Moreover, uptake of inulin as indicator of an increased endocytosis rate was enlarged. Electron micrograph analysis indicated a decrease of number and length of microvilli and a reduced oocyte surface area (Vasilets et al., 1990). The mouse retinal taurine transporter (TAUT) is also a target for PKC. Electrophysiological measurements

demonstrated that regulation of TAUT by PKC occurs largely by changes in the number of transporters present at the plasma membrane (Loo et al., 1996). In the case of the human cationic amino acid transporter isoforms hCAT-1 and hCAT-3, microscopic and Western blot analysis indicated that activation of PKC induced a reduction in surface expression paralleled with a decrease of hCAT mediated-transport (Rotmann et al. 2006; Rotmann et al 2004; Gräf et al., 2001). Similar findings were observed in the case of the sodium-dependent glutamate transporter EAAC1 (Trotti et al., 2001) and the human dopamine transporter hDAT1 (Zhu et al. 1997; Doolen et al, 2002). Likewise hSGLT1, activation of PKC had positive effects on the activity of the GABA transporter GAT1 by redistribution of transporter proteins from the cytosol to the plasma membrane (Corey et al., 1994). To summarize, PKC plays an important role in triggering trafficking processes in *X.* oocytes, albeit the response to PKC stimulation is not identical for all transporter proteins.

## 5.4 Regulation of Pept1 activity by substrate oversupply

In the present thesis, an oversupply of dipeptides evoked a profound decrease in transport activity and membrane density in *X.* oocytes overexpressing rPept1. Exposure of rPept1-expressing oocytes immediately after cRNA injection for 3 days to low concentrations of the dipeptide Gly-Gln resulted in a concentration dependent decline in transport currents that could be measured after the incubation period. To determine the effects of a shorter incubation period but higher substrate concentration rPept1-expressing oocytes were incubated in a nearly saturating concentration of Gly-Gln 3 days after cRNA injection, i.e. when the protein synthesis was nearly complete. After 2 hours incubation period, the peptide-induced transport currents decreased by about 60 %. Since the mean membrane capacitance remained unchanged, this decline most probably resulted from a reduction in transporter activity rather than from a decrease in the number of peptide transporters expressed in the membrane surface. To test the time dependency, incubation period was extended to 4 hours. Transport currents continued to decrease accompanied in this case by a decline in membrane capacitance by about 40 %. Since $K_m$ remained unchanged, while $V_{max}$ decreased, the changes were most likely caused by a decrease of transporter protein density in the plasma membrane. In favour of this assumption, immunofluorescence methods indicated a decline in fluorescence intensity of FLAG-labelled rPept1 proteins after 4 hours exposure to substrate, thus indicating a redistribution of transporter proteins from the membrane surface to intracellular compartments.

To test the possibility that the trigger mechanism for the above observations is the accumulation of dipeptides, experiments with the non-hydrolysable beta-lactam antibiotic cefadroxil were performed. Exposure of rPept1-expressing oocytes for 4 hours to cefadroxil decreased transport currents and membrane capacitance in a similar way as did Gly-Gln. However, unclear is whether the mechanisms inducing a downregulation in rPept1 activity after exposure to Gly-Gln and cefadroxil are the same. The experiments performed in Caco-2 cells (see chapter 4.5) rather indicated an apoptosis after long term treatment with cefadroxil, which was however, not the case for *X*. oocytes.

Inhibition of proteinase activity by simultaneous application of bestatin to the Gly-Gln-containing medium reduced membrane capacitance of rPept1 markedly after an incubation period of 4 hours. In contrast to the results observed after 4 hours exposure to Gly-Gln without bestatin, transport currents of rPept1 did not decrease but increase significantly. This observation is very surprising, since it could have been assumed that prevention of Gly-Gln hydrolysis resulted in a large increase in intracellular dipeptide concentration, which in turn blocked the inwardly-directed transport currents.

The first studies demonstrating a substrate-induced downregulation of transporter function in *X.* oocytes were published by Taylor (1996) and Gulley (2002) and their coworkers. Exposure of oocytes to a mixture of amino acids in saline evoked a downregulation of the Na-dependent amino acid transport systems $B^{0,+}$ and $X^{-}_{ag}$ whereas the Na-independent amino acid system were upregulated, thus, providing evidence of the existence of endogenous signalling mechanisms responsible for modulating the activity of amino acid transport systems in *X*. oocytes (Taylor et al., 1996). In another study, brief exposure (for 1 min once every 5 min for 1 hour) of *X.* oocytes expressing the human dopamine transporter (hDAT) to low micromolar concentrations of dopamine (3 µM, 10 µM), AMPH (10 µM) or tyramine (2 µM) resulted in a profound decline in transport currents accompanied by a retrieval of hDAT proteins from the cell surface. This observations were confirmed by Kahlig et. al (2003) by stable transfecting HEK-293 cells with a yellow fluorescent protein-tagged hDAT.

In contrast to the observations made in *X.* oocytes overexpressing rPept1, in Caco-2 cells, a prolonged exposure to dipeptides did not reduce but rather increased activity of Pept1. Previous studies with Caco-2 cells demonstrated that addition of the dipeptide Gly-Sar (10 mM) in the cell culture medium for 24 hours stimulates Gly-Gln uptake by about 71 %. Qualitative and quantitative analysis of Northern blots showed that this increase in

transport was accompanied by a nearly three-fold augmentation in the abundance of Pept1 mRNA. This stimulating effect on transport activity was not observed when incubation time was shortened to 2 hours (Thamotharan et al., 1998). Furthermore, studies by Walker et. al (1998) showed that exposure of Caco-2 cells for 3 days in peptide-rich medium (4 mM Gly-Gln) increased peptide transport activity 1.64 fold. The underlying mechanism for this upregulation in activity was an increase in Pept1 mRNA levels as well as in Pept1 protein expression.

In the present work exposure of Caco-2 cells for only 8 hours to various dipeptides (each 5 mM) resulted in a similar increase in peptide transport activity. Exposure to Gly-Sar and Gly-Gly, both robust and relatively slowly hydrolysed dipeptides (Adibi et Soleimanpour 1974; Walker et al, 1998), increased Gly-Sar uptake rate 1.72 respectively 2.04 fold. Addition of Gly-Gln, also a relative stable dipeptide but still hydrolysed by peptidases to some extent, resulted in an 1.41 fold stimulation of transport activity as compared to the control group.

In contrast, addition of Gly-Leu to the cell culture medium failed to stimulate Gly-Sar uptake. An amino acid analysis of the cytosol of Caco-2 cells did not detect measurable amounts of this dipeptide after 8 hours incubation period. This results are in accordance with previous studies demonstrating that Gly-Leu is a good substrate for intraluminal as well as membrane bound hydrolases (Adibi et Morse 1971; Adibi et Soleimanpour, 1974).

## 5.5 The fate of the intracellular absorbed dipeptides

In the present study, the results of an amino acid analysis of the cytosol of Caco-2 cells indicate that after 8 hours incubation, no measurable amounts of the dipeptide Gly-Gln were detected, though relative large amounts of the respective amino acids glycine and glutamine were found. Similar results were observed in *X.* oocytes after 4 hours exposure to Gly-Gln. Additional experiments in Caco-2 cells with the dipeptides Gly-Sar, Gly-Leu and Gly-Gly showed that all of these dipeptides, except for the very stable dipeptide Gly-Sar, were mostly hydrolysed intracellular during an incubation period of 8 hours.

Intracellular hydrolysis of the dipeptide Gly-Gln was effectively blocked by simultaneous addition of the proteinase inhibitor bestatin to the dipeptide-containing solution. Contrary, in the case of the dipeptide Gly-Leu, bestatin failed to prevent hydrolysis. This suggests a very fast hydrolysis of Gly-Leu, possibly even before it has entered the cell lumen. This would answer the question why exposure of Caco-2 cells to Gly-Leu did not have any stimulating effects on transport activity of Pept1.

## 5.6 *Xenopus* oocytes versus Caco-2 cells: Differences between both systems

Since the cytosolic pathways leading to the decline in transport activity of Pept1 in *X.* oocytes and to an increase in activity in Caco-2 cells are not known, the reason for the observed opposite regulatory mechanisms are still not understood. Unlike Caco-2 cells, *X.* oocytes do not possess endogenous Pept1-DNA, cRNA has to be injected into the oocytes and thus, RNA levels do not underlie changes as in Caco-2 cells.

Another difference is, that Caco-2 cells are polarized, enabling substrates i.e. dipeptides, amino acids, to leave the cell at the basolateral membrane. In oocytes, the absorbed substrate is very likely accumulated intracellular which in turn may activate further signalling pathways or cascades that in the end may trigger the endocytosis of rPept1 proteins.

In *X.* oocytes as well as in Caco-2 cells, prolonged exposure to dipeptides had profound, albeit contrary, effects on transport activity of Pept1 which might be caused either by the dipeptide itself or -due to hydrolysis- by changes of intracellular levels of free amino acids. In favour of the first hypothesis is the observation, that inhibition of hydrolysis of dipeptides by bestatin even enlarged stimulation of Gly-Sar uptake in Caco-2 cells and increased transport currents of rPept1-expressing oocytes. In contrast to this hypothesis are the results of the amino acid analysis of the cytosol of Caco-2 cells and rPept1-expressing oocytes. As already mentioned, the findings indicate that after an incubation period of 4 or hours the dipeptides are mostly hydrolysed to free amino acids. Experiments to investigate the effects of a direct injection of free amino acids into rPept1-expressing oocytes failed, since oocytes either did not survive the manoeuvre or died after a short incubation period (< 1 hour). As far as concerning Caco-2 cells, studies by Thamotharan et al. (1997) did not observe any stimulating effects on peptide transport activity when the free amino acids glycine and sarcosine were added to the cell culture medium for 24 hours.

In summary, it is still unclear, what mechanism triggers the stimulating effect on transport activity of Pept1 in Caco-2 cells. Though, the results in chapter 4.4 show that this increase seems to be caused by the insertion of newly synthesized transporter proteins into the cell membrane since disrupture of the Golgi complex by brefeldin A completely reversed the stimulating effect of Gly-Gln or Gly-Sar. These investigations are in accordance with a study by Walker et al. (1998) in which an increase in Pept1 protein expression by a factor of 1.72 (compared to the control group) was observed after stimulation of Caco-2 cells with 4 mM Gly-Gln for 3 days.

## 5.7 Regulation of Pept1 activity by intracellular acidification

Healthy, full-grown stage V/VI oocytes show an intracellular pH between 7.3 to 7.7 (Rodeau et al., 1998, Sasaki et al., 1992; Burckhardt et al., 1992, Busch et al., 1995; Miller et al. 1994, Thwaites et al., 1993 b). In this work, the results of the intracellular pH measurements were also in this range.

The driving forces for maintaining absorptive dipeptide uptake mediated by Pept1 are the membrane potential, the substrate and the proton gradient (pH difference between inside and outside). At least the results in chapters 3.8.2 and 3.6 indicated, that the first two parameters are unlikely to be the trigger mechanism in the substrate-induced downregulation of Pept1 activity in oocytes. The effects of pH changes on the functional activity of different transporters or channels have already been discussed (Miller et al., 1994; Thwaites et al., 1993 b; Xu et al., 2001). In Caco-2 cells, pH homeostasis is maintained by apically and basolaterally located $Na^+/H^+$ exchange systems. The $Na^+/H^+$-exchanger NHE3 seems to play an important role in maintaining dipeptide uptake across epithelial cells (Kennedy et al. 2002; Watanabe et al., 2005). Moreover, evidence for an interaction between Pept1 and NHE3 to mediate optimal dipeptide and $Na^+$ absorption across the apical membrane is provided. Kennedy et al. (2002) demonstrated that the uptake of dipeptides across the apical membrane of epithelial cells depended on extracellular $Na^+$ and was inhibited by inactivating NHE3 with the selective blocker S1611. Coinfection of Caco-2 cells with Pept1 and NHE3 resulted in a marked increase in $V_{max}$ of Pept1 that was about three times higher than in the absence of NHE3 (Watanabe et al., 2005).

In contrast, regulation of intracellular pH in *X.* oocytes is not clearly understood. The existence of an endogenous $Na^+/H^+$ antiporter has been reported but the contribution of this transporter to pH homeostasis in oocytes is unclear by now (Towle et al., 1991; Burckhardt et al., 1992). Besides, it has been shown that functional activity of this $Na^+/H^+$ exchanger is affected by treatment of oocytes with collagenase and decreases with the length of time after oocyte removal from the frog (Towle et al., 1991; Busch et al., 1998).

The uptake of dipeptides by Pept1 is accompanied by an influx of protons which might exceed the buffer capacitiy of the cell and in turn acidify the cytosol. Investigations using the pH indicator BCECF showed that a brief application of 20 mM Gly-Gln to the apical as well as basolateral membranes of Caco-2 monolayers resulted in an intracellular acidification (Thwaites et al., 1993 b). However, possibly long-term effects on transport activity of Pept1 due to the lowered $pH_i$ were not tested. However, in Caco-2 cells, unlike in oocytes, apical absorbed protons can leave the cell via basolateral located $Na^+/H^+$-

exchange systems thus, most likely, leaving intracellular pH relatively constant over longer periods.

To test if a moderate acidification of the cytosol has a direct effect on the rPept1-mediated transport under saturating substrate conditions, oocytes were acutely treated with sodium butyrate for 3 hours. This evoked an acidification similar to that observed in rPept1-expressing oocytes exposed for 4 hours to Gly-Gln (about 0.5 pH units), but in contrast to the dipeptide treatment, transport currents of Pept1 were not reduced. This observation shows that under saturating substrate conditions the pH gradient plays only a minor role as a driving force for the transport, but this does not exclude, however, the possibility, that a slow intracellular acidification induces processes that in turn, downregulate transport activity of Pept1.

Moreover, the results of the coexpression experiments support the role of intracellular acidosis in the substrate-induced downregulation of Pept1 activity. When rPept1 and hSGLT1 were coexpressed, uptake of sodium ions after exposure to α-MDG did not decrease but even increased transport activity of rPept1 markedly. On the other hand, after exposure to Gly-Gln, transport activity of hSGLT1 was affected only little whereas Pept1 activity decreased significantly about 60 % paralleled with a reduction in membrane capacitance.

Similar effects were observed after exposure of mPAT1- and hSGLT1-oocytes to glycine or α-MDG. Transport currents of mPAT1 declined about 40 % after exposure to glycine. However, activity of mPAT1 was not significantly enlarged after exposure to α-MDG, as in the case of rPept1. Transport currents of hSGLT1 decreased too, but in contrast to rPept1 or mPAT1, only slightly.

Surprisingly, exposure of oocytes coexpressing rPept1 and mPAT1 to cefadroxil or glycine did not only decrease transport activity of rPept1 (about 40 %) but also reduced transport currents of mPAT1 about the same degree in parallel with a significant decrease in membrane capacitance.

Assuming that the absorbed protons exceed the buffer capacity of the cytosol, the results prove the hypothesis that an intracellular overload of protons may have profound diminishing effects on both the function of the peptide transporter Pept1 and the amino acid transporter mPAT1 overexpressed in *X.* oocytes. The reduction of transport activity is, however, not the direct consequence of a reduced driving force, but could be triggered by an acidic pH in the microenvironment of these transporters. Furthermore, these investigations also suggest that the reduction in transport activity is limited to proton-

dependent transport systems whereas sodium-dependent systems, as in the case of hSGLT1, are not affected.

As already mentioned, in contrast to Caco-2 cells, hardly anything is known about the mechanisms maintaining $pH_i$ in *X.* oocytes, So far, it can not be excluded, that the observed decrease in transport activity is the result of an intracellular acidification and, furthermore, possibly is a phenomenon, specific for *X.* oocytes. In the end, to get more insight into these observations, a better understanding of pH homeostasis in *X.* oocytes requires further experiments.

# 6. Conclusion

For a long time, it has been believed that dietary proteins are absorbed exclusively in completely hydrolysed form as amino acids. In the early seventies the existence of a carrier-dependent system for dipeptides in mammalian intestinal cells has been proved that, in contrast to several known transport systems, was independent of sodium ions and was coupled to an inward current of protons. A further break through was reached in 1994 by cloning of the first mammalian intestinal peptide transporter Pept1 from human and rabbit. It became obvious that Pept1 plays a major role not only in the uptake of di- and tripeptides, but also several peptide-like drugs and pharmacologically active compounds are accepted and transported by the intestinal peptide transporter.

While strong emphasis was focused on determination of molecular structure, characteristics and function of this peptide transporter, by now, much less is known about regulation mechanisms of Pept1.

In 1998, studies in Caco-2 cells have shown for the first time that an oversupply of dipeptides for longer periods (24 hours to 3 days) resulted in a stimulation of Pept1 activity due to an enhanced abundance of Pept1 mRNA and protein expression, however, the pathway of this mechanisms was not found (Thamotharan et al., 1998; Walker et al., 1998).

The results of the previous study indicated a similar stimulation of transport activity of Pept1 in Caco-2 cells when the length of the incubation period was only 8 hours and when cells were pre-exposed to the dipeptides Gly-Gln, Gly-Sar or Gly-Gly, whereas no stimulating effect was observed by addition of Gly-Leu to the cell culture medium. Inhibition of peptidase activity with bestatin even enlarged the stimulatory effect, while it was fully blocked by disrupting the function of the golgi complex with brefeldin A, indicating that the insertion of newly synthesised proteins into the cell membrane has been suppressed.

One aim of the present work was to investigate whether *Xenopus* oocytes overexpressing r(abbit) Pept1 behave in a similar way as Caco-2 cells to a prolonged exposure to substrate. Electrophysiological measurements were used to determine transport currents and membrane capacitances of *X.* oocytes overexpressing rPept1, and these measurements were combined with immunofluorescence techniques.

The experiments presented evidence that insertion of peptide transporters into the membrane surface of oocytes resulted in an increase in membrane surface area

paralleled by a rise in dipeptide-induced transport currents with a slope of about 11 nF/100 nA transport current (at saturating substrate concentration).

However, in contrast to Caco-2 cells, exposure of *X.* oocytes directly after cRNA injection to various concentrations of the dipeptide Gly-Gln for 3 days did not increase, but markedly decreased transport activity of rPept1 in a concentration dependent manner. Furthermore, transport currents declined by about 60 % after exposure of rPept1-expressing oocytes 3 days after cRNA injection for 2 hours to Gly-Gln (5 mM) with no change in membrane capacitance. When incubation time was prolonged to 4 h, transport currents continued to decrease, but this time paralleled by a reduction in membrane capacitance by about 34 %. Immunofluorescent labelling of Pept1 confirmed that the reduction in surface area was due to a withdrawal of peptide transporter proteins from the oocyte membrane. A similar decrease in transport current and membrane capacitance was observed, when rPept1-expressing oocytes were exposed to the beta-lactam antibiotic cefadroxil or after activation of protein kinase C (PKC) by the phorbol esther PDBu. Treatment of rPept1-expressing oocytes with the highly-selective non-transported inhibitor Lys[Z($NO_2$)]-Pro did not affect transport activity, suggesting that not substrate binding but active transport of substrate into the cell is necessary to alter transport activity and surface expression of rPept1.

The role of the cytoskeleton was investigated by application of cytochalasin D (CD). CD inhibited for the largest part the decrease in transport activity of rPept1 observed after exposure to dipeptides or PKC stimulation. The selective PKC-inhibitor bisindolylmaleimide I blocked the PKC-stimulated endocytosis but failed to inhibit the substrate-induced downregulation of rPept1 activity.

The driving forces for maintaining absorptive dipeptide uptake mediated by Pept1 are the membrane potential, the substrate and the proton gradient. In search for the mechanism of the observed transporter downregulation a possible influence of these parameters in this process have been investigated. Experiments with the human sodium-dependent glucose transporter hSGLT1 showed that prolonged exposure of oocytes expressing this transporter leading to long lasting depolarisation of the cell membrane did not trigger endocytosis of transporter proteins. A reduction of the driving force by substrate accumulation could also be excluded, since amino acid analysis of the cytosol of the oocytes showed that the dipeptide Gly-Gln was completely hydrolysed during 4 hours incubation period (or even much faster, data not shown).

Intracellular pH ($pH_i$) measurement of rPept1-expressing oocytes indicated that after a incubation period of 4 hours in Gly-Gln $pH_i$ was lowered about 0.4 units. However, a fast depolarisation by the same value by addition of short chain fatty acids to the bathing

solution showed that at saturating substrate concentration this pH change does not have a direct effect on the transport current. However, coexpression experiments of rPepT1 with hSGLT1 or the proton-dependent amino acid transporter PAT1 from murine (mPAT1) support the hypothesis that long-lasting changes in $pH_i$ play a triggering role in the endocytosis of peptide transporters. Exposure of oocytes coexpressing rPept1 and hSGLT1 to dipeptides only reduced transport activity of rPept1, whereas activity of hSGLT1 was not affected. When rPept1 and mPAT1 were coexpressed, transport activity of both proton-dependent transporters declined significantly after exposure to dipeptides or amino acids, thus suggesting, that reduction of pHi, due to an intracellular accumulation of protons, selectively affects proton-dependent transporters but has no impact on the sodium-dependent transporter hSGLT1. By now, little is known about the regulation of $pH_i$ in *Xenopus* oocytes. The existence of an endogenous sodium/proton exchanger has been proven (Towle et al., 1991; Burckhardt et al., 1992), but its role in maintaining $pH_i$ in oocytes is still unclear. Also unknown are the impacts of a prolonged intracellular acidification on transport properties of transporter proteins. The results in this work suggest that the cotransported protons are accumulated intracellularly which may exceed the buffer capacity and acidify the cytosol, followed by the activation of yet unknown mechanisms that trigger the endocytosis of the transporter proteins.

# 7 Abbreviations

| | |
|---|---|
| ATP | adenosine-5-triphosphate |
| BBMV | brush border membrane vesicles |
| BGT1 | betain GABA transporter |
| BFA | brefeldin A |
| BIM | bisindolylmaleimide 1 |
| BSA | bovine serum albumin |
| CD | cytochalasin D |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| $C_m$ | membrane capacitance |
| cRNA | copy ribonucleic acid |
| DAT | dopamine transporter |
| dNTP | deoxyribonucleotidetriphosphate |
| ddNTP | dideoxyribonucleotidetriphosphate |
| DEPC | diethylpyrocarbonate |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |
| dsDNA | double-stranded DNA |
| EAAC1 | sodium-dependent glutamate transporter 1 |
| E. coli | Escherichia coli |
| EDTA | ethylenediaminetetraacetic acid |
| GABA | gamma-aminobutyricacid |
| GAT1 | gamma-aminobutyricacid transporter 1 |
| GLUT5 | glucose uniporter 5 |
| Gly-Gln | glycylglutamine |
| Gly-Gly | glycylglycine |
| Gly-Leu | glycyleucine |
| Gly-Sar | glycylsarcosine |
| h | hour(s) |
| HCl | hydrochloricacid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| hCAT | human cationic amino acid transporter |
| hPept1 | human peptide transporter 1 |
| hSGLT1 | human sodium-dependent glucose transporter |
| $I_{max}$ | maximal transport velocity |
| IV | current/voltage |
| $K_m$ | Michael-Menten affinity constant |
| LiCl | lithium chloride |
| MES | 2-(N-morpholino)ethanesulfonic acid |
| min | minute(s) |
| ml | millilitre |
| µg | microgram |
| µl | microlitre |
| mPAT1 | murine proton-dependent amino acid transporter 1 |
| mRNA | messenger ribonucleic acid |
| mV | mili volt |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| nF | nano farad |
| ng | nanogram |
| NHE | sodium/proton exchanger |

| | |
|---|---|
| nm | nanometer |
| PAT | proton-dependent amino acid transporter |
| PBS | phosphate buffer solution |
| PDBu | phorbol-12,13-dibutyrate |
| PHT | peptide/histidine transporter |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PCR | polymerase chain reaction |
| rENAC | rat epithelial sodium channel |
| rPept1 | rabbit peptide transporter 1 |
| rpm | rounds per minute |
| s | second(s) |
| SAP | shrimp alkaline phosphatase |
| SGLT | sodium-dependent glucose transporter |
| TAUT | taurine transporter |
| TEVC | two-electrode-voltage-clamp |
| TMD | transmembrane domain |
| U | unit |

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*-Sri Chinmoy*

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