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Transport of Charged Dipeptides by the Intestinal H^+/Peptide Symporter PepT1 Expressed in *Xenopus laevis* Oocytes

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Abstract. The cloned intestinal peptide transporter is capable of electrogenic H^+-coupled cotransport of neutral di- and tripeptides and selected peptide mimetics. Since the mechanism by which PepT1 transports substrates that carry a net negative or positive charge at neutral pH is poorly understood, we determined in *Xenopus* oocytes expressing PepT1 the characteristics of transport of differently charged glycylpeptides. Transport function of PepT1 was assessed by flux studies employing a radiolabeled dipeptide and by the two-electrode voltage-clamp-technique. Our studies show, that the transporter is capable of translocating all substrates by an electrogenic process that follows Michaelis Menten kinetics. Whereas the apparent $K_{0.5}$ value of a zwitterionic substrate is only moderately affected by alterations in pH or membrane potential, $K_{0.5}$ values of charged substrates are strongly dependent on both, pH and membrane potential. Whereas the affinity of the anionic dipeptide increased dramatically by lowering the pH, a cationic substrate shows only a weak affinity for PepT1 at all pH values (5.5–8.0). The driving force for uptake is provided mainly by the inside negative transmembrane electrical potential. In addition, affinity for proton interaction with PepT1 was found to depend on membrane potential and proton binding subsequently affects the substrate affinity. Furthermore, our studies suggest, that uptake of the zwitterionic form of a charged substrate contributes to overall transport and that consequently the stoichiometry of the flux-coupling ratios for peptide: H^+/H_3O^+ cotransport may vary depending on pH.

Key words: Intestinal peptide transporter — Expression — Substrate specificity — Two-electrode voltage-clamp technique

Introduction

Di- and tripeptides in the intestinal lumen derive from the hydrolysis of dietary and endogenous proteins by a concerted action of pancreatic proteases and brush border membrane bound peptide hydrolases. Short chain peptides released are either hydrolyzed to the constitutent amino acids or are taken up in intact form into epithelial cells by specific peptide transport systems in the apical membrane. The intestinal peptide carriers have recently been cloned from rabbit (PepT1) [1, 5] and human (hPepT1) [6] small intestinal cDNA libraries. Injection of the rabbit intestinal peptide transporters cRNA into *Xenopus laevis* oocytes induces a transport activity that is characterized as Na^+, K^+ and Cl^- independent but electrogenic as a consequence of peptide/H^+ cotransport. PepT1 appears to have a broad substrate specificity accepting di- and tripeptides [1, 5, 6] as well as a variety of peptide mimetics including β-lactam antibiotics [1, 5, 10, 13, 14] and selected angiotensin-converting enzyme inhibitors [1]. Although some functional as well as preliminary structural informations of the intestinal peptide transporters are available [4, 5, 8], the operational mode of PepT1 is poorly understood. There are several thousand possible peptides which could serve as substrates for the transport system including a variety carrying a net negative or positive charge at physiological pH. Concerning the fundamental questions whether and how PepT1 transports these differently charged substrates, only preliminary information is available [2, 9, 11]. We have employed flux studies with radiolabeled D-phenylalanyl-L-alanine and the two-electrode voltage-clamp technique in *Xenopus* oocytes expressing PepT1 to gain information on binding and transport of differently charged dipeptides.

Materials and Methods

Peptides were purchased from Sigma (Deisenhofen, FRG). Custom-synthesized D-[^{3}H]-phenylalanine-L-alanine (^{3}H-D-Phe-Ala) with a specific activity of 9 Ci/mmol was obtained from Zeneca (UK).

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Methods

Xenopus laevis Oocytes and Transport Assay

Oocyte preparation and handling have been described previously [1]. The oocytes were injected with 50 nl of H_2O (controls) or 5 ng/50 nl of Pep T1 complementary RNA (cRNA). Three days post injection uptake of ^{3}H-D-Phe-Ala was assessed under standard assay conditions in a buffer composed of (in mM): 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 5 N-2-hydroxyethylpeperazine-N′-2-ethane-sulfonic acid (HEPES)/Tris or 5 2-(*N*-morpholino)ethanesulfonic acid (MES)/Tris to allow changes in medium pH from 5.5 to 8.0. Oocytes displaying at least a 10-fold increase of peptide uptake over water-injected controls were used for both flux measurements and electrophysiology. Kinetics of ^{3}H-D-Phe-Ala influx was measured for 10 min of incubation in the presence of increasing concentrations of D-Phe-Ala (0.025 to 5.0 mM) and uptake rates in water injected control oocytes were substracted. Inhibition of ^{3}H-D-Phe-Ala influx (0.25 μM) in the presence of competing substrates was determined at pH 8.0, 7.4, 6.5 and 5.5 in the presence of 1 mM of either glycyl-L-glutamine (Gly-Gln), glycyl-L-aspartate (Gly-Asp) or glycyl-L-lysine (Gly-Lys).

Electrophysiology

A conventional two-electrode voltage-clamp technique was applied to characterize responses in current (*I*) to substrate addition in oocytes injected with 5 ng transporter cRNA as described previously [11]. Steady-state current-voltage (*I–V*) relationships were measured in the absence and the presence of different peptides with water-injected oocytes serving as controls. Membrane potential in oocytes was held at −60 mV and stepped symmetrically to test potentials from −150 to +50 mV by 500 msec rectangular voltage pulses and current recordings were obtained during the last 100 msec. Inward currents in voltage clamped oocytes as a function of substrate concentration were measured at pH values of 8.0, 7.4, 6.5 and 5.5.

Titration Curves and Dissociation/association Profiles

To determine the percentage of the respective ionic species of Gly-Asp, Gly-Gln and Gly-Lys present at the different pH values as used in the experiments we obtained the dipeptides titration curves. This was done by titrating 20 ml of 5 mM dipeptide dissolved in the same buffers as used in the experiments (without Tris or Mes) and subsequent addition of 100 μl of 0.1 N NaOH or HCl respectively. pK_a values were determined after plotting the pH measured as a function of meq OH^-/H^+ added and subsequent calculation of the turning points using INPLOT. The percentage of the various substrate species as a function of pH was calculated according to the Henderson-Hasselbalch equation as described previously [13].

Calculations

All calculations (linear as well as nonlinear regression analysis) were performed by using INPLOT, statistical analysis by using INSTAT (GraphPAD, Los Angeles, CA). Flux studies as well as most of the electrophysiological experiments were carried out with 5–8 oocytes from at least two separate batches and results are presented as the means ± SEM. For selected studies representative data obtained in individual oocytes are presented. Comparative analysis of kinetic parameters for the different substrates was performed in experiments utilizing the same batch of oocytes.

Abbreviations

PepT1, rabbit H^w/oligopeptide transporter 1 (hPep T1, human), ChoCl, choline chloride, Gly-Gln, glycyl-L-glutamine, Gly-Asp, glycyl-L-aspartate, Gly-Lys, glycyl-L-lysine, Lys-Lys, L-lysyl-L-lysine, Glu-Glu, L-glutamyl-L-glutamate, V_m, oocyte membrane potential.

Results

Characteristics of D-Phe-Ala Transport into Oocytes Expressing PepT1

Injection of the transporters cRNA into oocytes resulted in pH dependent influx of ^{3}H-D-Phe-Ala uptake (50 μM) that exceeded at pH 6.0 influx into water injected control oocytes several fold (Fig. 1, left panel, *inset*). D-Phe-Ala uptake as a function of substrate concentration ([S]) at pH 6.0 displayed saturation kinetics with an apparent $K_{0.5}$ value of 1.15 ± 0.21 mM (Fig. 1, left panel). When in the same batch of oocytes D-Phe-Ala evoked inward currents were recorded as a function of [S] (Fig. 1, right upper panel), a saturation kinetics was obtained with an almost identical apparent $K_{0.5}$ value of 1.23 ± 0.18 mM (Fig. 1, right lower panel). In addition, a significant linear correlation ($P < 0.001$) is obtained when inward currents are plotted against the corresponding influx rates as measured under indentical experimental conditions. These functional data demonstrate that dipeptide transport into oocytes expressing Pep T1 is pH dependent at low substrate concentration, saturable and electrogenic as a consequence of dipeptide/H^+ cotransport.

Interaction of Charged Dipeptides with ^{3}H-D-Phe-Ala Uptake

To investigate whether and how differently charged substrates interact with PepT1, we first determined to which extent the uptake of ^{3}H-D-Phe-Ala (25 μM) into oocytes is inhibited by Gly-Asp, Gly-Gln and Gly-Lys at pH values of 8.0, 7.4, 6.5 and 5.5. As shown in Fig. 2, Gly-Gln displays at all pH values the strongest inhibitory potency among the three substrates. In contrast, 1 mM Gly-Asp showed no interaction with PepT1 at pH 8.0 but inhibited ^{3}H-D-Phe-Ala uptake at pH 7.4. Gly-Lys reduced D-Phe-Ala influx by 23% at pH 8.0 and by 31% at pH 7.4. When pH was lowered to ⩽6.5 Gly-Asp reduced influx of the labeled substrate by more than 75%, whereas Gly-Lys inhibited influx significantly at pH 6.5 (50%; $P < 0.001$) but not at pH 5.5. These data show, that the interaction of differently charged dipeptides with the transporters substrate binding site is strongly dependent on external pH. Whereas the relative affinity of the zwitterionic Gly-Gln was only modestly affected by pH, Gly-Asp affinity increased significantly with decreasing pH. The apparent affinity of Gly-Lys was highest at pH 6.5 but was in general low when compared to the other substrates.

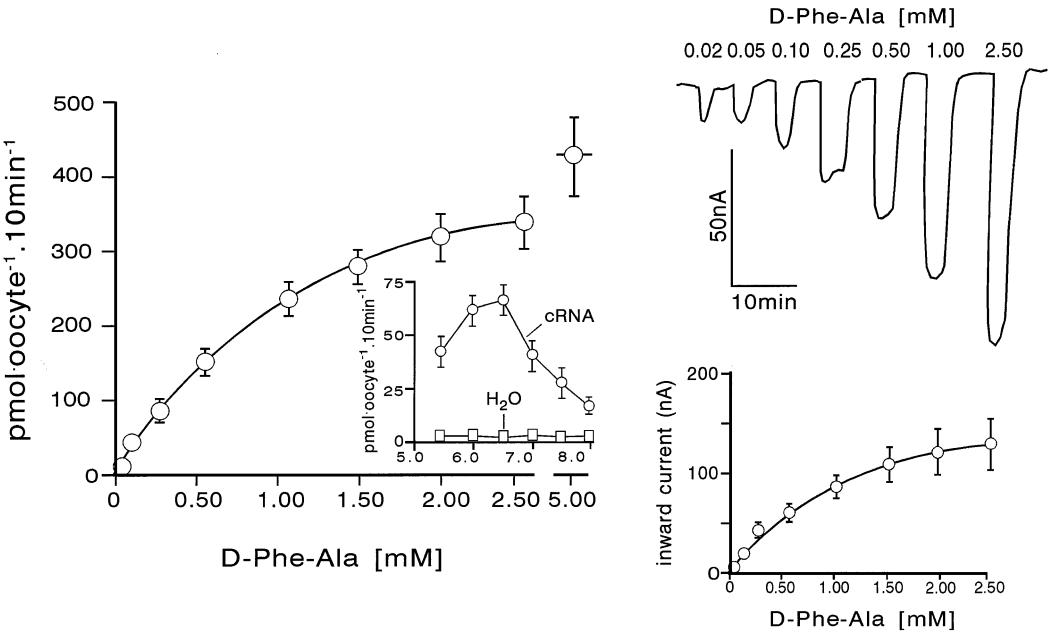

Fig. 1. *Left panel:* Concentration-dependent kinetics of ^{3}H-D-Phe-Ala influx into oocytes expressing rabbit PepT1. Three days post injection of 5 ng of PepT1-cRNA uptake of 0.025 to 5.0 mM D-Phe-Ala was measured at pH 6.0. Corresponding uptake rates for D-Phe-Ala in water-injected control oocytes served as controls and were substracted. Data were fitted to a Michaelis-Menten kinetics by nonlinear regression analysis by the least squares method ($K_{0.5}$: 1.15 ± 0.21 mM; V_{max}: 628.6 ± 37.6 pmol · oocyte^{-1} · 10 min^{-1}) and are presented as the mean ± SEM. *Inset:* pH dependence of D-Phe-Ala uptake into oocytes expressing PepT1. Uptake of 25 μM ^{3}H-D-Phe-Ala determined at buffer pH 5.5 to pH 8.0. Uptake rates of control oocytes were substracted. Data are presented as mean ± SEM. *Right upper panel:* Substrate-evoked inward currents in an oocyte expressing PepT1 as a function of D-Phe-Ala concentration. Three days after injection of 5 ng of PepT1-cRNA individual oocytes were perfused with increasing D-Phe-Ala concentrations at pH 6.0 and substrate-evoked inward currents were recorded while the membrane potential was clamped to −60 mV. Water injected control oocytes showed no current response when perfused with D-Phe-Ala. *Right lower panel:* Concentration dependent kinetics of inward currents generated by superfusion of oocytes expressing PepT1 with increasing amounts of D-Phe-Ala at pH 6.0. Oocytes were clamped to −60 mV and substrate evoked inward currents were plotted according to Michaelis-Menten ($K_{0.5}$: 1.23 ± 0.18 mM; V_{max}: 97 ± 33 nA).

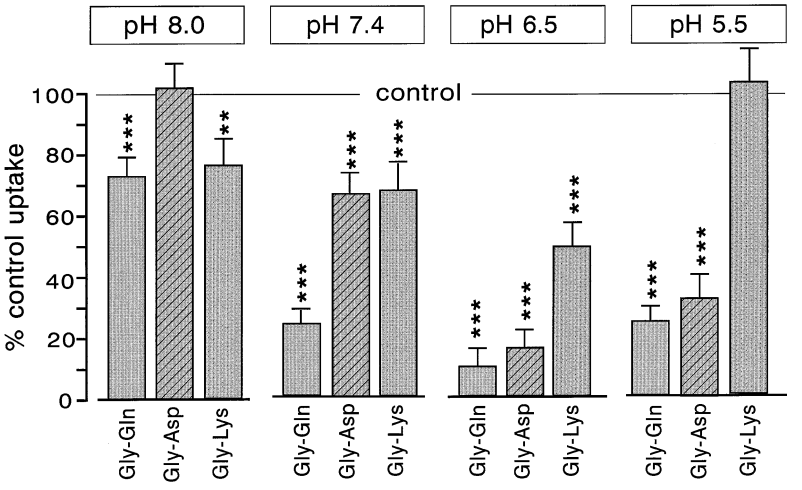

Fig. 2. Inhibition of ^{3}H-D-Phe-Ala influx into oocytes expressing PepT1 by selected dipeptides (Gly-Gln, Gly-Asp, Gly-Lys) as a function of pH. Uptake of 25 μM ^{3}H-D-Phe-Ala was measured three days after cRNA injection in the absence (control) or presence of 1 mM of the dipeptides. Values are expressed as the means ± SEM. ***Significantly different from control ($P < 0.001$), **($P < 0.01$).

Inward Currents in Response to Combined Dipeptide Perfusion

Although the inhibition data already suggested that differently charged substrates compete with D-Phe-Ala for a common binding site on PepT1, in a next series of experiments we determined whether substrate-evoked inward currents were also mediated by a common substrate binding site. For this purpose the two-electrode voltage-clamp technique was applied to measure inward currents in oocytes clamped to −60 mV at pH 6.5 in response to the three differently charged dipeptides. As shown in

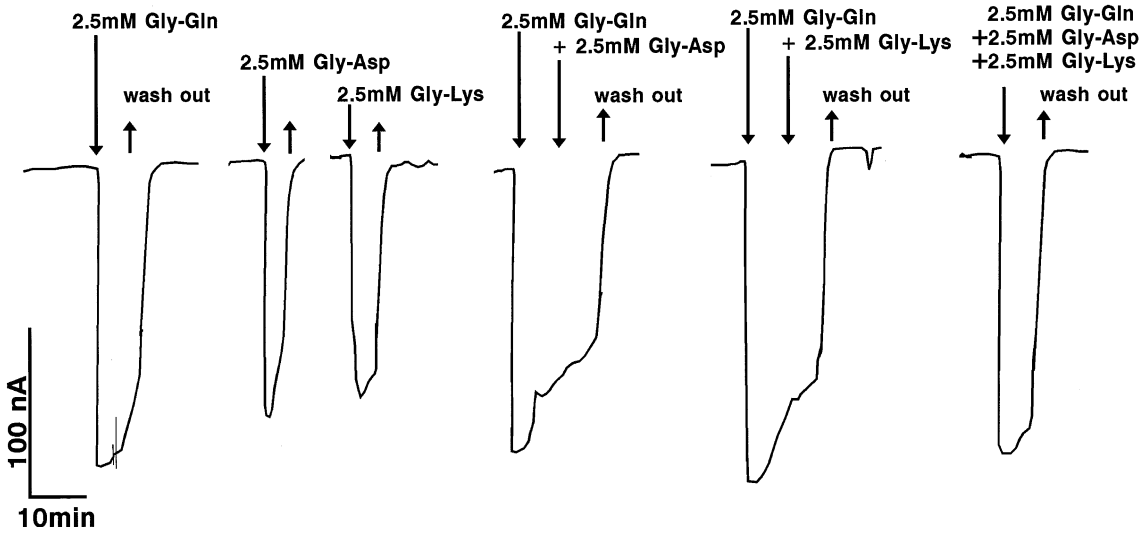

Fig. 3. Representative inward currents in oocytes expressing PepT1 evoked by superfusion with 2.5 mM Gly-Gln, Gly-Asp or Gly-Lys. In addition, current responses as a consequence of superfusion with 2.5 mM Gly-Asp or Gly-Lys subsequent to 2.5 mM Gly-Gln and to a simultaneous application of the three differently charged dipeptides are shown. Membrane potential was clamped to −60 mV and currents were recorded at pH 6.5 by using a conventional two-electrode voltage-clamp.

Fig. 3 the three substrates at a concentration of 2.5 mM caused similar currents irrespective of their charge. Perfusion of oocytes with 2.5 mM Gly-Gln evoked mean inward currents of 131 ± 22 nA that did not change significantly when Gly-Gln concentration was increased to 10 mM. Addition of 2.5 mM Gly-Asp or Gly-Lys subsequently to Gly-Gln perfusion or a combined substrate perfusion with Gly-Gln, Gly-Asp and Gly-Lys caused inward currents that were similar to those obtained for Gly-Gln perfusion alone. In all cases, currents returned to baseline valueswhen substrates were washed out (Fig. 3). Since substrate-evoked currents did not show any additive response when perfused simultaneously or consecutively, it is concluded that all dipeptides indeed seem to interact at a single binding site on PepT1.

Inward Currents Generated by Gly-Asp, Gly-Gln and Gly-Lys as a Function of Substrate Concentration and pH

Currents generated by influx of the different peptides (oocytes clamped to −60 mV) were recorded at pH 6.5 in response to increasing substrate concentrations (0.1 to 5 mM). All three substrates displayed saturation kinetics (Fig. 4*A*) with similar maximal currents (I_{max}) but different response at low substrate concentrations. When similarly the substrate evoked currents were measured at pH 5.5 and 7.4, the saturation kinetics obtained allowed a comparative analysis of apparent affinity constants ($K_{0.5}$) and apparent I_{max} values (Fig. 4*B*). Inspection of Fig. 4*B* reveals that substrate-evoked currents differ in their dependence on extracellular pH. Comparing the current responses for the different peptides at saturating substrate concentrations and different pH shows that in case of the zwitterionic peptide (Gly-Gln) currents were only moderately affected by pH, with lowest currents generated at pH 5.5. In contrast, currents generated by Gly-Asp increased almost fivefold by lowering pH from 7.4 to 5.5. Currents evoked by Gly-Lys were similar at pH 7.4 and 6.5 but were reduced by almost 50% at pH 5.5. At pH 8.0 Gly-Asp did not cause any inward currents and Gly-Gln evoked currents were too low to be analyzed (Table). Nevertheless, under these obviously unfavorable conditions for electrogenic uptake of zwitterionic and anionic peptides, the cationic Gly-Lys caused currents of 180 ± 22 nA at a membrane potential of −60 mV. The divergent dependence of I_{max} on extracellular pH in case of the cationic Gly-Lys and the anionic Gly-Asp was found to be even more pronounced when the dianionic glutamyl-glutamate (Glu-Glu) and the dicationic lysyl-lysine (Lys-Lys) were investigated (Table). Whereas Lys-Lys caused highest inward currents at pH 8.0, it did not cause any detectable currents at pH 5.5. In contrast, Glu-Glu failed to induce a positive charge transfer at pH 8.0 and pH 7.4 but generated high inward currents of 185 ± 25 nA at pH 5.5.

That not only the I_{max} values, but also the apparent affinities of the differently charged dipeptides are affected by external pH is shown in Fig. 4*B*. Whereas the high affinity of the zwitterionic Gly-Gln remained essentially unaffected by pH (pH 5.5: 0.139 ± 0.031 mM, pH 6.5: 0.173 ± 0.042 mM, pH 7.4: 0.156 ± 0.037 mM), the app. $K_{0.5}$ values for Gly-Asp and Gly-Lys were found to be extremely dependent on pH. In case of the cationic substrate, a very low affinity of 2.911 ± 0.522 mM was determined at pH 5.5, that increased to 0.567 ± 0.115 mM

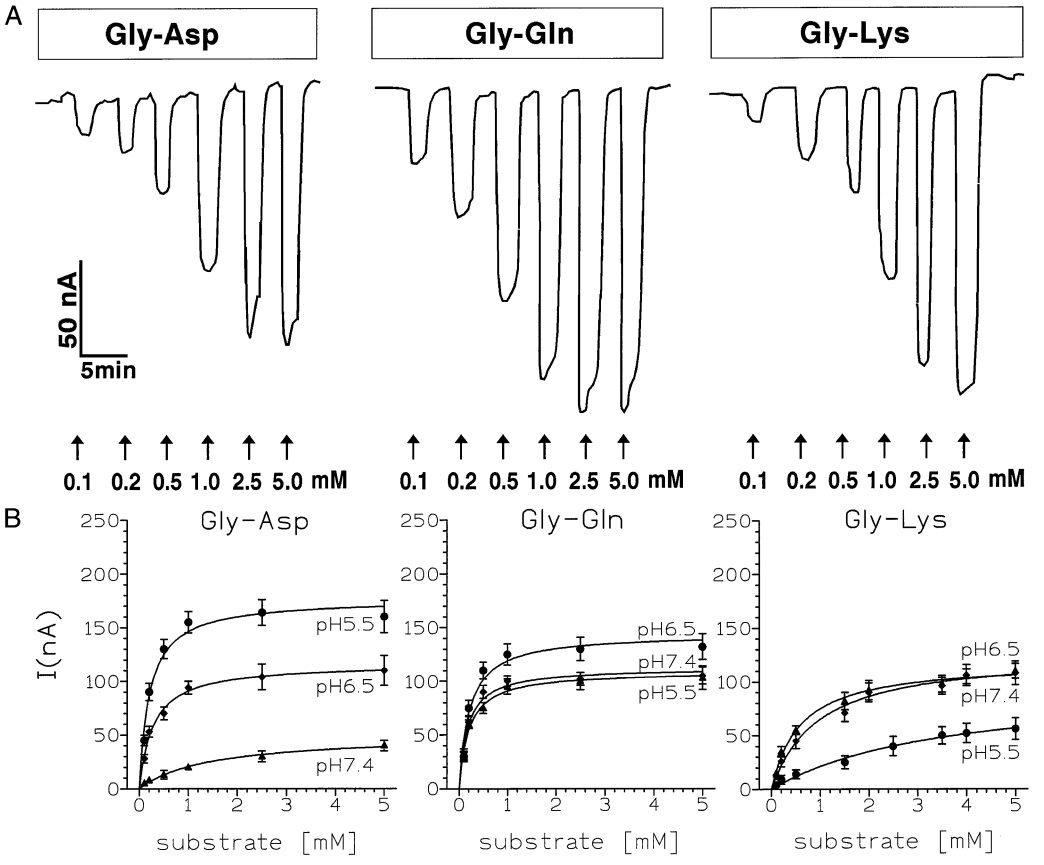

Fig. 4. (*A*) Inward currents in individual oocytes expressing PepT1 as evoked by Gly-Asp, Gly-Gln or Gly-Lys as a function of dipeptide concentration (pH 6.5). Membrane potential was clamped to −60 mV and oocytes were superfused consecutively with increasing concentrations of the peptide. After recording the currents at a given substrate concentration, oocytes were perfused with a dipeptide free buffer at pH 6.5 until current returned to baseline values followed by infusion of the next solution containing a higher substrate concentration. (*B*) Concentration-dependent kinetics of current responses to perfusion of oocytes with Gly-Asp, Gly-Gln or Gly-Lys (0.1 to 5 mM) at pH 5.5, pH 6.5 and pH 7.4. Oocytes expressing PepT1 were superfused with increasing dipeptide concentrations at the different pH values and resulting inward currents measured at a holding potential of −60 mV were fitted by nonlinear approximation according to the least squares method to a Michaelis-Menten equation.

Table. Current responses (in nA) in voltage-clamped oocytes expressing PepT1 when perfused with selected differently charged dipeptides as a function of pH_{out}

	pH 5.5	pH 6.5	pH 7.4	pH 8.0
Lys-Lys	0	65 ± 17	155 ± 16	192 ± 29
Gly-Lys	58 ± 11	109 ± 25	117 ± 30	180 ± 22
Gly-Gln	97 ± 15	131 ± 22	102 ± 36	2 ± 1
Gly-Asp	181 ± 12	100 ± 19	31 ± 11	0
Glu-Glu	185 ± 25	48 ± 9	0	0

Maximal inward currents (I_{max}) in oocytes expressing PepT1 obtained by superfusion with 5 mM or 10 mM of the individual dipeptides. I_{max} values were measured at a holding potential of −60 mV using the two-electrode voltage-clamp. Data are presented as the mean ± SEM of 6 to 8 oocytes.

at pH 6.5 and 0.392 ± 0.104 mM at pH 7.4. The opposite was observed for Gly-Asp; were app. $K_{0.5}$ decreased from 1.341 ± 0.221 mM at pH 7.4 to 0.186 ± 0.033 mM at pH 6.5 and 0.135 ± 0.041 mM at pH 5.5.

I_{MAX} AS A FUNCTION OF $[H^+]_{out}$ AND V_m

To analyze the pH dependence of peptide binding and transport in more detail we determined the substrate-evoked currents (at 2.5 mM) as a function of the apparent extracellular proton concentration in oocytes clamped to membrane potentials of ±0, −60 or −100 mV. From the corresponding steady state *I–V* relationships at pH 8.0, 7.4, 6.5, 5.85 and 5.5, currents were replotted as a function of apparent $[H^+]_{out}$ and different membrane potentials. As shown in Fig. 5, in the absence of a membrane potential, inward currents as a function of $[H^+]_{out}$ displayed saturation kinetics for $[H^+]_{out}$ for all three substrates with Hill-coefficients not different from 1 (1.18 ± 0.21). Half maximal transport in the absence of a membrane potential was achieved at $[H^+]_{out}$ concentrations of approximately 200 nM in the presence of Gly-Gln, 100 nM in case of Gly-Lys and 400 nM in case of Gly-Asp. Maximal currents at zero membrane potential reached approximately 50 nA for all substrates, indicating that

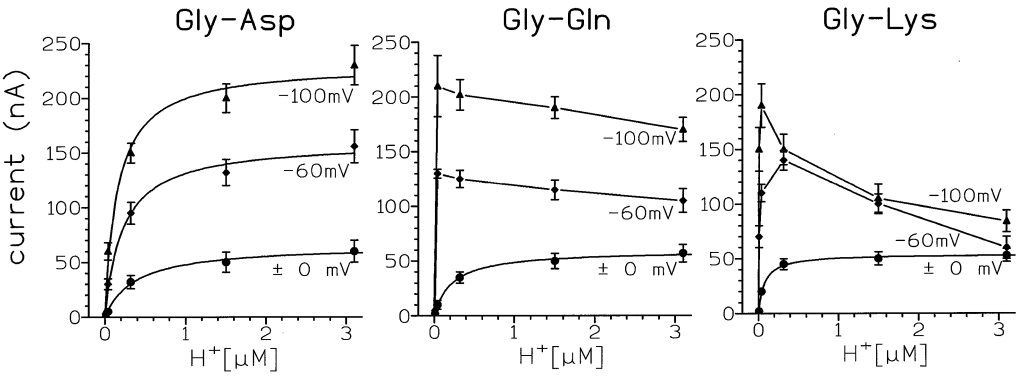

Fig. 5. Substrate-evoked inward currents in oocytes clamped to membrane potentials of 0, −60 and −100 mV and perfused with buffers of different pH containing 2.5 mM of the dipeptides. Current responses were taken from steady-state current-voltage (*I–V*) relationships in oocytes where membrane potential was stepped symmetrically to potentials between +50 and −150 mV. Substrate-dependent inward currents were replotted as the difference measured in the absence and presence of 2.5 mM dipeptide. Data are presented as the mean ± SEM of 6 to 8 oocytes.

when only the substrate and proton gradients provide the driving force, charge movement mediated by PepT1 was essentially independent of the kind of substrate and its charge.

When the membrane potential is clamped to −60 or −100 mV, the apparent affinity of H^+_{out} for its interaction with PepT1 increases significantly in the presence of all substrates (Fig. 5).[6] Because Gly-Gln and Gly-Lys induced maximal currents already at pH 7.4, the apparent $K_{0.5}$ for $[H^+]_{out}$ has to be considerably lower than 40 nM at inside negative membrane potentials. In contrast, the $[H^+]_{out}$ dependence of the Gly-Asp mediated currents showed a significantly higher $K_{0.5}$ for $[H^+]_{out}$ of 210 ± 31 nM at −60 mV and 153 ± 23 nM at −100 mV respectively. When $[H^+]_{out}$ was increased from 316 nM (pH 6.5) to 3.1 μM (pH 5.5) in the presence of a hyperpolarized membrane potential, Gly-Gln induced currents were almost unaffected whereas those generated by Gly-Lys and Gly-Asp displayed opposite changes. Gly-Asp mediated currents slightly increased, Gly-Lys evoked currents declined with increasing proton concentration.

From the data obtained on $[H^+]_{out}$ interaction with a proposed binding site on the transporter, it appears that initial proton interaction with PepT1 is a prerequisite for any substantial PepT1 activity. In the presence of an inside negative membrane potential this transport activation is achieved even in the absence of a proton gradient, except in case of Gly-Asp. Moreover, it needs to be emphasized, that although the maximal currents for the different substrates were found to be almost identical (255 ± 43 nA), these I_{max} values were obtained under quite different experimental conditions. Gly-Asp evoked highest I_{max} was measured at −100 mV and pH 5.5, those for Gly-Gln and Gly-Lys were obtained at pH 7.4 (Fig. 5).

Substrate Affinities as a Function of pH and V_m

Since membrane potential has a pronounced effect on inward currents, as shown in Fig. 5, we investigated in more detail the dependence of substrate affinity on V_m at different pH values. Steady-state current-voltage relationships were recorded in individual oocytes perfused at pH 7.4, 6.5 and 5.5 and in the presence of increasing substrate concentrations. From the corresponding *I–V* relationships apparent $K_{0.5}$ values were derived for the different substrates at membrane potentials changed from +50 to −150 mV. When $K_{0.5}$ values were plotted as a function of V_m (Fig. 6), it is observed that Gly-Gln affinities were less affected by V_m at any given pH than those of Gly-Asp and Gly-Lys. Nevertheless, $K_{0.5}$ values for Gly-Gln showed a dependence on V_m by decreasing almost tenfold when the membrane potential was depolarized (e.g., at pH 6.5 from $K_{0.5}$ 0.145 mM at −150 mV to 0.013 mM at ±0 mV). This increase in affinity as a consequence of membrane depolarisation was even more pronounced in case of Gly-Asp and much more pH dependent. Hyperpolarization of the oocyte membrane decreased the affinities more than tenfold at any pH, whereas lowering pH increased affinity at any V_m (Fig. 6). In contrast, affinities for Gly-Lys increased by increasing pH from 5.5 to 7.4 but simultaneously affinities were reduced by hyperpolarization of $V_m \leqslant -50$ mV. These data demonstrate the complex interrelationship between membrane potential and $[H^+]_{out}$ in affecting the affinities by which differently charged dipeptides interact with the substrate binding site of PepT1.

Discussion

Hydrolysis of dietary proteins in the gastrointestinal tract produces a large amount and a huge variety of short chain peptides. Not only will these peptides vary with respect to net charge and solubility, they also cover a wide range of molecular weights from 96.2 Da (di-Gly) to 522.6 Da (tri-Trp). A fundamental question that has not been investigated in detail is how the peptide transporter handles charged substrates. More than 20% of all

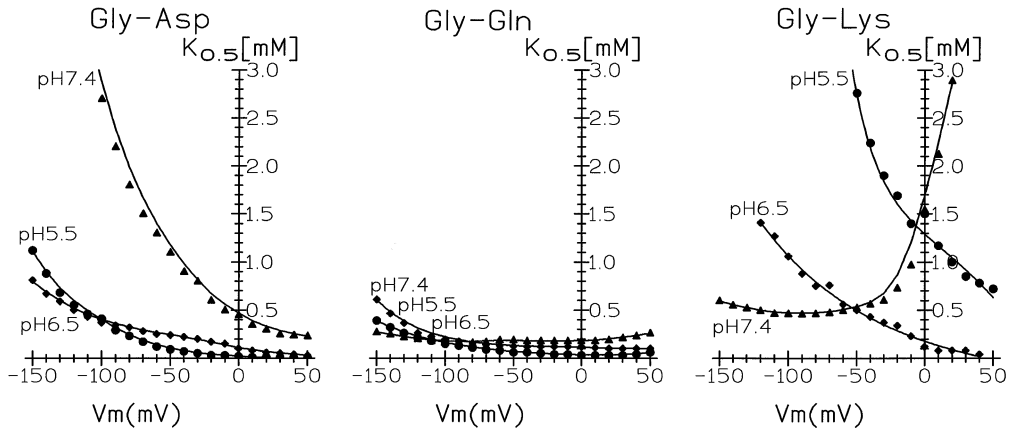

Fig. 6. Apparent substrate affinities as a function of pH and membrane potential. Oocytes expressing PepT1 were perfused at pH 5.5, pH 6.5 or pH 7.4 and in the presence of increasing substrate concentrations (0.1 to 5 mM). At each substrate concentration, steady state *I–V* relationships were recorded by the two-electrode voltage-clamp technique and currents obtained were replotted as a function of substrate concentration. Data were fitted to a Michaelis-Menten kinetics by nonlinear regression analysis by the least squares method and apparent $K_{0.5}$ values of the different peptides were calculated for each individual membrane potential.

possible di- and tripeptides carry net negative or positive charge at physiological pH and competition experiments have established that a number of charged substrates do interact with the transporters substrate binding sites [3, 5, 10, 13, 14].

The present study was designed to elucidate the characteristics by which PepT1 transports differently charged dipeptides. By measuring substrate evoked currents in *Xenopus* oocytes expressing PepT1 we could demonstrate that all dipeptides, regardless of their net charge, are transported electrogenically. We found that dipeptide transport obeys Michaelis Menten type saturation kinetics with apparent $K_{0.5}$ values and I_{max} values differently affected by V_m and pH for neutral and charged substrates. We furthermore showed that maximal transport velocity in case of neutral substrates is only dependent on membrane potential and not on pH. In case of charged substrates a complex interrelationship between membrane potential and $[H^+]_{out}$ determines substrate affinity and consequently the transport rates.

Variable Flux Coupling Ratios and Transport of the Noncharged Substrate Species

The striking finding that all substrates regardless of their net charge are transported electrogenically addresses the question of whether this is a consequence of variable flux coupling rates for substrate and H^+/H_3O^+ translocation. Although Fei et al. [5] and Mackenzie et al. [7] concluded that transport of a zwitterionic substrate (Gly-Sar) occurs with a 1:1 H^+/peptide flux coupling stoichiometry, electrogenic transport of charged substrates requires a different stoichiometry for H^+ movement when a similar transport mode is assumed.

Rheogenic Gly-Asp uptake can only be achieved by a H^+ translocation rate that exceeds the substrate flux rate to compensate for the charge of the substrate. Although a 2:1 coupling ratio could explain electrogenic Gly-Asp flux at low pH easily, it has to be taken into account, that the substrates charge changes simultaneously by alterations in pH. To be able to calculate the percentage of the different ionic species present at a given pH, we obtained titration curves for all three substrates in media at the same ionic strength as the buffers used in the experiments. From the titration curve of Gly-Asp we calculated pK_a values of 2.81, 4.45 and 8.60 and therefore at pH 6.5 1.0% and at pH 5.5 8.2% of the substrate are in the uncharged form (Fig. 7). At saturating dipeptide concentrations as used (>2.5 mM) this is equivalent to >200 μM of substrate in its neutral form at pH 5.5. Assuming that the apparent affinity of the zwitterionic form of Gly-Asp ($Gly\text{-}Asp^{\pm}$) is not very much different from that of the Gly-Gln, one could predict, that the Gly-Asp evoked currents under these conditions are totally (or at least mostly) generated by transport of the neutral form. With a $K_{0.5}$ for Gly-Asp found to be ⩽100 μM (at pH 5.5 and −60 mV), maximal transport rates at this pH could result from transport of the neutral form by a similar or identical flux coupling ratio as for zwitterionic dipeptides such as Gly-Gln or Gly-Sar. This hypothesis is further supported by the dependence of the Gly-Asp $K_{0.5}$ on membrane potential. Similar to the zwitterionic Gly-Gln, affinity of Gly-Asp at pH 6.5 and 5.5 is high and is only modestly affected by alterations in membrane potential between ±0 and −100 mV. Shifting pH to 7.4 (only 0.1% of Gly-Asp is uncharged) at inside negative membrane potentials causes a tenfold increase in $K_{0.5}$, which indicates a very low affinity of the negatively charged substrate species for its interaction with the binding site on PepT1. However, it has to be taken into account that increasing $[H^+]_{out}$ also activates trans-

port of Gly-Asp by increased proton binding to PepT1 and consequently by increasing the transport capacity. A low extracellular pH therefore could affect transport of an anionic substrate by (a) an increased binding affinity of the anionic peptide species after protonation of one or more functional groups in the substrate binding domain and/or by (b) an increased affinity of the now also present zwitterionic form of the substrate. Inward currents evoked by Gly-Asp could consequently always represent the sum of transport of the anionic form with at least two protons and/or the zwitterionic species occurring by a predicted 1:1 flux coupling ratio representative for neutral substrates. According to this concept, the proposed dual transport mode would contribute to overall currents to a different extent depending on $[H^+]_{out}$ and membrane potential.

A similar dual transport mode could be postulated for the cationic Gly-Lys. Again, pH and V_m would be determinants for both the coupling rate and the changes in substrate affinity. The assumption of a Gly-Lys$^+$:H$^+$ coupling ratio identical to that of zwitterionic substrates (e.g., 1:1), would result in a net substrate flux for Gly-Lys half of that of Gly-Gln at the same I_{max}. The Gly-Lys$^+$:H$^+$ coupling ratio of 1:1 with additive charge movement (50% substrate, 50% proton) could explain the significant inward currents measured under conditions were inhibition of transport of D-Phe-Ala by Gly-Lys was extremely low (*see* Fig. 2). However, changes in the percentage of the Gly-Lys carrying no net charge when the pH is elevated above pH 6.5 are not negligible. Increasing pH to 7.4 generates 13.1% of uncharged Gly-Lys and at pH 8.0 more than 37% of the substrate is in the zwitterionic form (Fig. 7). Transport of the zwitterionic form of Gly-Lys therefore could also contribute significantly to total currents. The hypothesis that the neutral Gly-Lys species is the preferred transported form is supported by the observation that highest maximal transport rates are obtained at very low $[H^+]_{out}$ and negative inside V_m. Similarly, highest substrate induced inward currents were observed for the dicationic Lys-Lys only at pH $\geq$ 7.4.

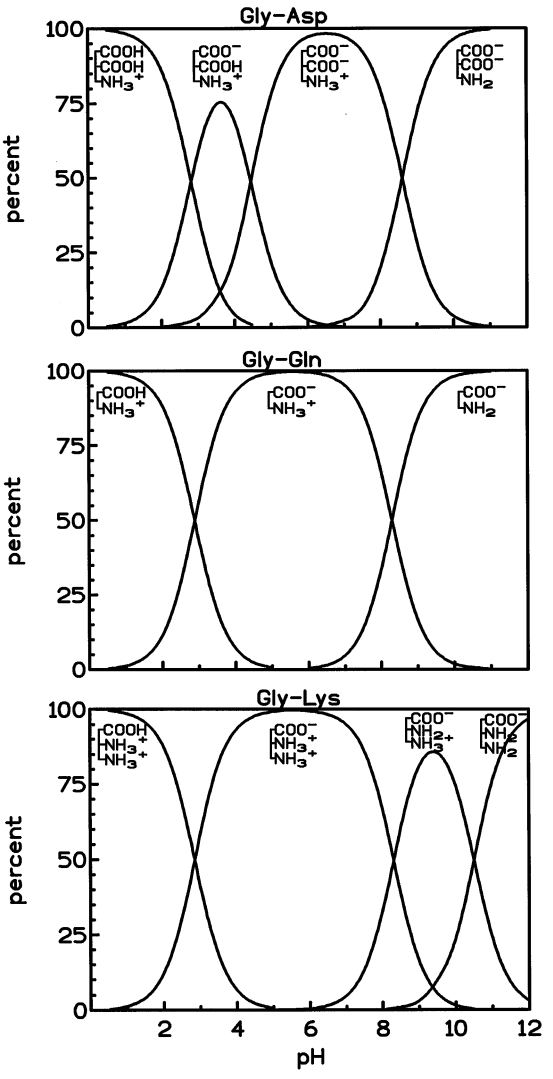

Fig. 7. Percentage of ionic species of Gly-Asp, Gly-Gln and Gly-Lys as a function of pH.

Effects of Membrane Potential on Substrate Affinity and Transport Velocity

The membrane potential plays an important role for maximal transport capacity of the various dipeptides tested. This becomes evident from Fig. 5, where a more than 20-fold increase in Gly-Gln (2.5 mM) evoked currents at pH 7.4 is observed when V_m is decreased from ±0 mV to −100 mV. That this increase in current is not a consequence of an altered substrate affinity becomes evident by inspection of Fig. 6. Gly-Gln affinity is extremely high and 2.5 mM substrate always causes saturation. In the case of zwitterionic substrates therefore, I_{max} is solely dependent on membrane potential and essentially unaffected by pH. In contrast, for the charged substrates adverse affinity effects counteract the transporters capability to reach maximal velocity. As demonstrated, the I_{max} for Gly-Asp increases severalfold at inside negative V_m by reducing pH_{out} below 7.4 and this is paralleled by an increase in substrate affinity at more acidic pH. Gly-Lys evoked currents on the opposite decrease significantly under these conditions. This obviously results from a markedly reduced affinity for binding of the charged Gly-Lys form to PepT1 and could be a consequence of an increased competition between Gly-Lys$^+$ and H^+/H_3O^+ at the proton binding site of PepT1 at low pH_{out}. This hypothesis is supported by the observation that in case of Gly-Lys an increase in $[H^+]_{out}$ from 40 nM to $\leq$1 μM at −60 mV and even more pronounced at −100 mV causes a significant reduction of transport

activation by H^+, which is paralleled by the marked loss in substrate affinity. This phenomenon could also be responsible for the small currents generated by Gly-Lys at hyperpolarized membrane potentials (i.e., at −100 mV) and pH 5.5, that are much lower than those evoked by the other substrates.

Effects of Membrane Potential and Substrate on H^+ Interaction with PepT1

The lack of a sigmoidal dependence of substrate evoked currents on external proton concentration, as shown in Fig. 5, suggests that all three peptides are transported by a 1:1 flux coupling ratio. It is pertinent to note that even the Hill coefficients obtained for Gly-Asp mediated currents as a function of $[H^+]_{out}$ (Fig. 5) never exceeded 1.25 ± 0.13 neither at low nor at high inside negative V_m. Although the corresponding Hill coefficients for H^+ activation could not be precisely determined in the presence of Gly-Lys or Gly-Gln at inside negative V_m, since transport was already fully activated at pH 7.4, our data do not provide any evidence for a sigmoidal activation of PepT1 by$[H^+]_{out}$. This suggests that nocooperative proton binding sites with different affinities are involved in $[H^+]_{out}$ activation of PepT1, but it does not exclude a priori multiple binding sites with similar or identical $K_{0.5}$ values for H^+/H_3O^+ binding.

The finding that the affinity for H^+_{out} interaction with its binding site is dependent on V_m confirms recent observations on H^+ activation of Gly-Sar influx into oocytes expressing hPepT1 [7]. Extending this study, we now show that H^+ binding is affected by charged substrates and that H^+ binding alters the substrate affinities of charged peptides. In case of anionic substrates H^+ binding affinity is reduced at low $[H^+]_{out}$ whereas high concentrations of a cationic substrate impare H^+ interaction at its binding site at low pH. This observation suggests that the proton binding sites in PepT1 are within the substrate binding pocket or in a close proximity. In addition, the extreme pH dependence of the different transport parameters strongly suggests His-residues to play a pivotal role in the phenomenons observed.

The pre-steady-state currents generated by hPepT1 shown in the above-mentioned paper give evidence for H^+ binding to hPepT1 as the initial step in an ordered process of peptide translocation [7]. Our data support this finding by demonstration of a very high affinity (app. $K_{0.5} < 20$ nM) for H^+ binding to rabbit PepT1 when an inside negative membrane potential is imposed. Under physiological conditions therefore most of the transporters H^+ binding sites are already saturated even in the absence of a significant proton gradient.

In conclusion, our studies show that affinity for dipeptide interaction with the substrate binding site is affected by H^+ binding to PepT1 and by changes in membrane potential. The alterations in substrate affinity by pH and V_m are much more pronounced in the case of charged than in the case of zwitterionic substrates. This appears to be due to a generally lower substrate affinity of the charged compounds and by changes in the percentage of zwitterionic form present by altering $[H^+]_{out}$. The neutral species of a charged dipeptide shows increased affinity for interaction with PepT1 and consequently increases the maximal transport rate.

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