Electrophysiological analysis of the function of the mammalian renal peptide transporter expressed in *Xenopus laevis* oocytes

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1. To gain information on the mode of operation of the renal proton-coupled peptide transporter PepT2, voltage clamp studies were performed in *Xenopus laevis* oocytes expressing the rabbit renal PepT2.

2. Using differently charged glycyl-dipeptides we show that PepT2 translocates these dipeptides by an electrogenic pH-dependent process that is essentially independent of the substrate net charge. The apparent substrate affinities are in the micromolar range (2–50 μM) between pH 5·5 and 7·4 and membrane potentials of ±0 to −50 mV.

3. Maximal substrate-evoked inward currents (I_{max}) are affected by membrane voltage (V_m) and extracellular pH (pH_o). Potential-dependent interactions of H^+/H_3O^+ with PepT2 seem to be mediated by a single low affinity binding site and PepT2 remains pH dependent at all voltages.

4. The effects of voltage on apparent I_{max} and substrate affinity display an inverse relationship. As V_m is altered from −50 to −150 mV substrate affinities decrease 10- to 50-fold whereas apparent I_{max} increases almost 10-fold.

5. Even at saturating H^+/H_3O^+ and dipeptide concentrations the I–V curves did not show saturation at negative membrane potentials, suggesting that other steps in the reaction cycle and not the ligand affinity changes are rate limiting. These are possibly the conformational changes of the empty and/or loaded transporters.

6. These findings demonstrate that not only substrate affinities but also other kinetic characteristics of PepT2 differ markedly from those of the intestinal peptide transporter isoform PepT1.

Cellular peptide uptake is a ubiquitous phenomenon since bacteria, fungi, plants and animals, including mammals, express functionally similar proton-dependent oligopeptide transporters (for reviews see Matthews, 1991; Steiner, Naider & Becker, 1995). These carriers are required for utilization of peptides as a source for amino acids and nitrogen (Daniel, 1996). Human, rabbit and rat peptide transporters have been cloned recently from cDNA libraries of intestine (PepT1 series: Boll, Markovich, Weber, Korte, Daniel & Murer, 1994; Fei *et al.* 1994; Liang *et al.* 1995; Saito, Okuda, Terada, Sasaki & Inui, 1995) and kidney cortex (PepT2 series: Miyamoto, Ganapathy & Leibach, 1986; Liu *et al.* 1995; Saito *et al.* 1995; Boll *et al.* 1996). Kidney brush-border membranes contain two distinct peptide transport systems with different kinetic characteristics (Silbernagl, Ganapathy & Leibach, 1987; Daniel, Morse & Adibi, 1991) most probably represented by PepT1 and PepT2. Whereas PepT1 is a low affinity high capacity system, PepT2 represents a high affinity low capacity phenotype. Di- and tripeptides filtered in the glomerulum or released by hydrolysis from larger peptides can therefore be reabsorbed efficiently in the tubulus, thus loss of amino acid nitrogen can be prevented.

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This manuscript was accepted as a Short Paper for rapid publication.

PepT1 and PepT2 translocate di- and tripeptides, as well as peptidomimetics, electrogenically by coupling of substrate transport to movement of H^+/H_3O^+ down an electrochemical gradient. Although the mode of operation of PepT1 in particular has recently been studied with respect to handling of differently charged peptides by a number of investigators (Boll *et al.* 1996; MacKenzie, Fei, Ganapathy & Leibach, 1996; Amasheh *et al.* 1997; Steel, Nussberger, Romero, Boron, Boyd & Hediger, 1997), essentially nothing is known about the transport mode of PepT2.

Since PepT2 shows only a low identity and in certain protein regions even a low homology to PepT1, it can be assumed that the characteristics of substrate translocation are also considerably different. We therefore applied the two-electrode voltage clamp technique to oocytes of the South African clawed toad (*Xenopus laevis*) expressing rabbit renal PepT2 to determine the nature of peptide-evoked inward currents. Representing the large variety of potential substrates, we have focused on transport characterization of differently charged glycyl-L-dipeptides. Recently, a similar characterization of the intestinal isoform PepT1 under identical experimental conditions has been performed (Amasheh *et al.* 1997).

METHODS

Oocyte preparation and injection

Female *Xenopus laevis* were purchased from African Xenopus Facility (Knysna, South Africa). Toads were anaesthetized in 0·22% Tricaine (Sigma), lobes of ovary were removed and after suture of the abdomen animals were allowed to recover. Oocyte preparation, storage and injection techniques were performed as reported recently (Boll *et al.* 1994; Amasheh *et al.* 1997).

Solutions

For all experiments, Mes- and Hepes-containing buffer solutions with different substrate concentrations ranging from 10 μM to 2·5 mM and different pH values ranging from 5·5 to 7·4 were used. The composition was (mM): NaCl, 100; KCl, 2; $CaCl_2$, 1; MgCl, 1; either Mes or Hepes, 5; pH was adjusted after addition of the substrates by adding Tris or Mes/Hepes. Oocytes were stored in a medium containing (mM): NaCl, 90; KCl, 1; $CaCl_2$, 2; Hepes, 5; sodium pyruvate, 2·5. The medium was supplemented with penicillin (20 mg l^{-1}) and streptomycin (25 mg l^{-1}). All compounds, if not stated otherwise, were purchased from Sigma.

Voltage clamp measurements

The conventional two-electrode voltage clamp technique was performed as described before (Weber, Schwarz & Passow, 1989).

Calculations

Results are expressed as means ± S.E.M. Kinetic analysis of transport currents was performed using InPlot (GraphPad, San Diego, CA, USA).

RESULTS

Current responses to substrates carrying different net charge

The addition of neutral (Gly–Gln), mono-anionic (Gly–Asp) or mono-cationic (Gly–Lys) dipeptides to oocytes expressing PepT2 always resulted in significant pH-dependent inward currents (I) under voltage clamp conditions (Fig. 1*A*). In contrast, in water-injected control oocytes no peptide-induced currents could be detected (not shown). Apparent maximal substrate-evoked currents (I_{max}; at saturating dipeptide concentrations) were essentially independent of the substrate charge in the extracellular pH (pH_o) range tested (not shown). Steady-state I–V relationships recorded during transport of differently charged substrates were

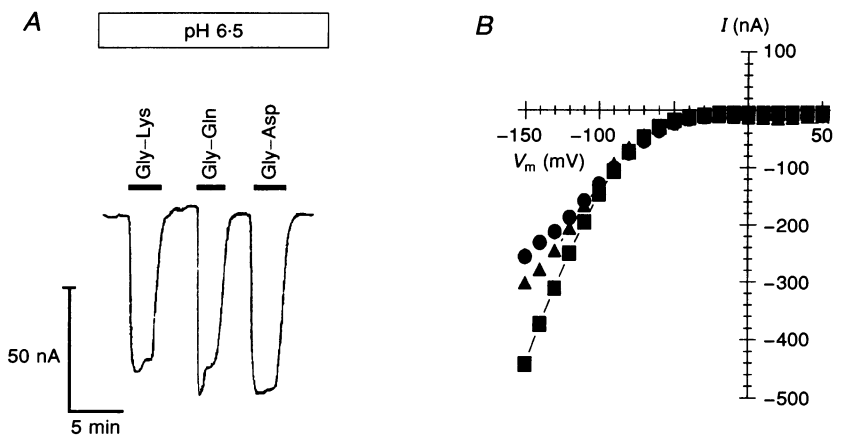

Figure 1

A, representative inward currents in oocytes expressing PepT2 evoked by superfusion with differently charged dipeptides. Three days post injection of 5 ng PepT2-cRNA, oocytes were clamped to −60 mV and superfused with 2·5 mM Gly–Lys, Gly–Gln or Gly–Asp at pH 6·5. *B*, recordings of I–V relationships in oocytes superfused with differently charged dipeptides. V_m was stepped symmetrically to test potentials between −150 and +50 mV in steps of 10 mV and current responses in the presence of 2·5 mM Gly–Lys (●), Gly–Gln (■) and Gly–Asp (▲), subtracted by the current in the absence of peptide were plotted as a function of V_m at pH 6·5.

similar in the physiological range of the endogenous membrane potential. Figure 1*B* shows the typical *I–V* relationship of an oocyte perfused with Gly–Gln, Gly–Lys and Gly–Asp. In the voltage range +50 to −20 mV inward currents did not respond significantly to alterations in membrane potential (V_m), whereas a weak potential dependence was observed in the physiological range −20 to −60 mV. At hyperpolarized membrane potentials (−60 to −150 mV), however, inward currents increased significantly and showed a linear dependence on V_m.

Current reponses as a function of substrate concentration, pH_o and V_m

Single oocytes were clamped to −60 mV and perfused with solutions containing increasing concentrations of the dipeptides. For all substrates, clamp current increased as a function of substrate concentration according to Michaelis–Menten kinetics. The $K_{0.5}$ of Gly–Gln-induced currents at pH 6·5 was 7·63 ± 1·72 μM. Whereas the $K_{0.5}$ for Gly–Asp-induced currents was similar (4·4 ± 1·3 μM), that of Gly–Lys was significantly lower (86·6 ± 33·4 μM).

Steady-state *I–V* relationships obtained during perfusion of oocytes with different substrate concentrations allowed the apparent substrate affinities to be determined as a function of V_m and pH_o. Figure 2*A* shows the apparent substrate $K_{0.5}$ values as a function of V_m at pH_o 5·5, 6·5 and 7·4. In the potential range ±0 to −50 mV only minor differences in apparent affinities could be observed at different pH values, whereas hyperpolarized potentials caused a dramatic decrease in affinity by up to 100-fold for all substrates when compared with that at ±0 mV.

For determination of the responses of inward currents to extracellular H^+/H_3O^+ concentration ($[H^+/H_3O^+]_o$), V_m was clamped to ±0 mV, −60 or −100 mV in the presence of the different dipeptides at saturating substrate concentrations. Since affinity of all substrates decreased at hyperpolarizing V_m, a substrate concentration of 2·5 mM was used to prevent kinetics being affected by reduction of $K_{0.5}$. As shown in Fig. 2*B*, in the presence of a membrane potential, currents increased with a hyperbolic function when $[H^+/H_3O^+]_o$ was increased. Whereas at ±0 mV the H^+/H_3O^+ affinity could

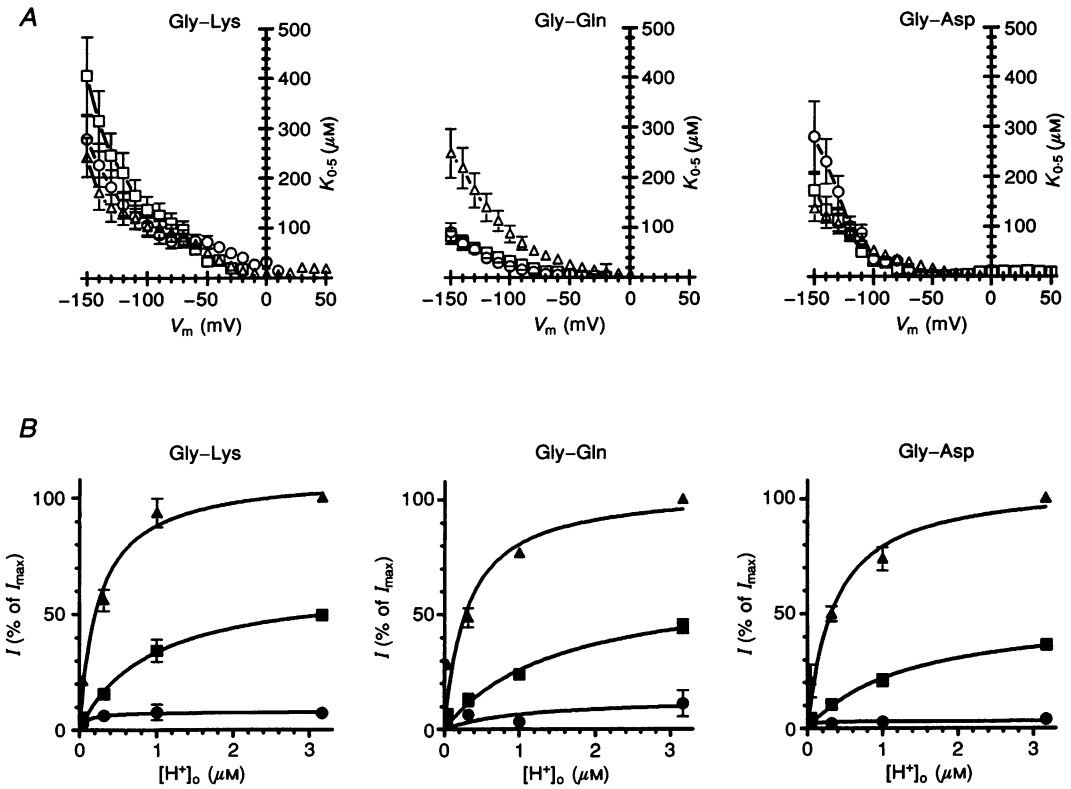

Figure 2

A, apparent substrate affinities for Gly–Lys, Gly–Gln and Gly–Asp as a function of V_m and pH_o. Oocytes were superfused with increasing concentrations of the different dipeptides (0·1 to 2·5 mM) at pH 5·5 (△), 6·5 (□) and 7·4 (○) and steady-state currents were replotted as a function of substrate concentration and fitted according to a Michaelis–Menten function to determine apparent $K_{0.5}$ for each substrate at a given V_m. *B*, effect of apparent $[H^+]_o$ and V_m on inward currents at saturating substrate concentrations. The oocytes were superfused with buffers of pH 5·5, 6·0, 6·5 or 7·4 containing 2·5 mM substrate. Current responses at ±0 (●), −60 (■) and −100 mV (▲) were taken from steady-state *I–V* measurements. Currents are fitted as a function of $[H^+]_o$ according to Michaelis–Menten kinetics. Data are presented as the means ± S.E.M. of 3–5 oocytes. Currents are expressed as a percentage of maximal response in an individual oocyte when compared with the current recorded at −100 mV.

not be determined due to extremely low transport currents, H^+/H_3O^+ affinity increased, irrespective of the substrate charge, from $1{\cdot}3 \pm 0{\cdot}25\ \mu$M (mean of all substrates) at -60 mV to $0{\cdot}31 \pm 0{\cdot}05\ \mu$M after V_m was jumped to -100 mV. At saturating proton and dipeptide concentrations I_{max} values were similar for Gly–Lys, Gly–Gln and Gly–Asp but increased almost linearly the more negative V_m became (Fig. 3).

These findings suggest that (a) interaction of H^+/H_3O^+ with the transporter protein occurs via a single binding site the affinity of which increases as the membrane potential hyperpolarizes, (b) H^+/H_3O^+ binding is essentially independent of the substrate type and (c) the apparent I_{max} of the transporter remains pH dependent at all voltages.

DISCUSSION

The renal peptide transporter PepT2 provides an efficient route for uptake of amino acids in peptide-bound form. Its very high affinity for di- and tripeptides consisting of L-α-amino acids ensures an almost complete recovery of peptides from the tubular fluids even when their concentration in the tubule is very low.

In the present study we applied the two-electrode voltage clamp technique to determine PepT2-mediated inward currents in response to alterations in V_m, pH_o and substrate concentration in *Xenopus laevis* oocytes expressing the renal transporter isoform. In addition we assessed the transport characteristics for glycyl-dipeptides carrying different net charges at physiological pH.

The comparative analysis of substrate-evoked inward currents revealed that PepT2 transports dipeptides, regardless of their net charge, with almost identical characteristics. Affinities for dipeptide interaction with the substrate binding site of PepT2 are essentially independent of extracellular H^+/H_3O^+ concentration and remain high ($2\ \mu M < K_{0.5} < 100\ \mu M$) until the membrane potential is hyperpolarized. Affinities for interaction of H^+/H_3O^+ with PepT2 are also independent of the substrate charge but increase 4-fold as V_m becomes more negative. Apparent I_{max} values are identical (at saturating concentrations of both ligands) even when differently charged dipeptides are translocated. I_{max} values of PepT2 are clearly a function of the proton-motive force. Since I–V curves did not show saturation at negative membrane potentials we have to postulate that other steps in the reaction cycle rather than changes in ligand affinities are rate limiting. These are most probably the conformational changes of the empty carrier and/or its relocation to the external membrane side.

When comparing the transport characteristics of PepT2 with those of PepT1, as determined under similar experimental conditions with the same substrates (Amasheh *et al.* 1997), we can conclude that (a) PepT2 displays substrate affinities of one to two orders of magnitude higher, (b) H^+/H_3O^+ interaction with PepT2 is, in contrast to that with PepT1, independent of substrate charge and occurs with a low affinity, (c) apparent I_{max} values for PepT2, in contrast to PepT1, are also independent of substrate charge, and (d) I_{max} obtained for PepT2 evoked by all substrates remain pH dependent at any given V_m in contrast to the pH independence of those for PepT1 (Amasheh *et al.* 1997).

The finding that inward currents for PepT2 are qualitatively and quantitatively similar regardless of the net charge of the substrate at a given pH addresses the possibility of a variable flux coupling ratio for H^+/H_3O^+-dependent peptide transport. The capability of peptide transporters to electrogenically transport mono- and divalently charged peptides has recently been studied in the case of PepT1 by three independent groups (MacKenzie *et al.* 1996; Amasheh *et al.* 1997; Steel *et al.* 1997). Whereas for zwitterionic substrates electrogenic transport based on a 1 : 1 flux coupling ratio was postulated by all groups, electrogenic transport of cationic and anionic dipeptides was explained by transport of only the non-ionized species and/or by variable proton : peptide stoichiometries. For anionic substrates a 2 : 1 and for cationic dipeptides a 1 : 1 proton : peptide coupling ratio has been postulated based on measurements

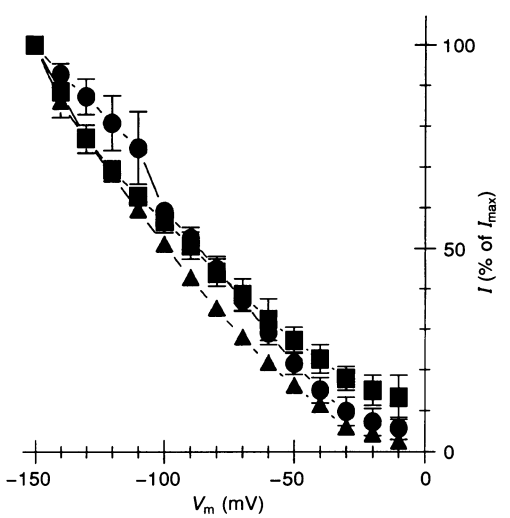

Figure 3. Comparison of maximal H^+ induced inward currents at saturating substrate concentrations as a function of V_m

Oocytes were perfused with buffers of pH 5·5, 6·0, 6·5 or 7·4 containing 2·5 mM Gly–Gln (■), Gly–Lys (●) or Gly–Asp (▲). Maximal inward currents at variable $[H^+]_o$ were obtained by non-linear regression analysis of the kinetics obtained by the least-squares method. Currents are expressed as a percentage of maximal response obtained at -150 mV.

of substrate-induced changes in membrane potential and intracellular pH (Steel *et al.* 1997) in individual oocytes expressing PepT1. Others proposed the counterflow of OH^- during electrogenic proton-coupled influx of a mono-anionic dipeptide to explain the similarities in charge and proton transfer rates of anionic and neutral dipeptides (MacKenzie *et al.* 1996). Lastly, transport of mainly the zwitterionic forms of charged substrates was proposed as the mechanism to explain the substrate-specific pH dependence of transport currents (Amasheh *et al.* 1997).

Here we show for PepT2 that the characteristics of both peptide and H^+/H_3O^+ interaction with the transporter are almost independent of the net charge of peptides. Due to the qualitative similarities in the transport kinetics we assume, as in our conclusion in the case of PepT1, that mainly (or only) the zwitterionic substrate species of a charged peptide is transported by a 1 : 1 flux coupling ratio. In the case of PepT2, kinetic analysis of $[H^+]_o$-dependent inward currents at saturating substrate concentrations did not provide any evidence for a Hill coefficient significantly larger than 1 (0·83–1·17) for any of the substrates. This lack of evidence suggests that there are no multiple H^+ binding sites with different affinities and favours the hypothesis of a fixed coupling ratio for transport of the substrate species carrying no net charge. There is evidence that a variety of other solute transporters also have the capability to transport anionic as well as cationic substrates electrogenically with similar kinetics. Recently this has been demonstrated for example for the *Arabidopsis thaliana* H^+/amino acid transporter AAP1/NAT2. Kinetics of electrogenic transport of differently charged amino acids were found to be very similar and explained by uptake of only the zwitterionic form of the substrates (Boorer, Frommer, Bush, Kreman, Loo & Wright, 1996). In the case of PepT2 the weak pH dependence of dipeptide affinities may be due to the very high substrate affinites. Even low concentrations of the zwitterionic form of a substrate would quickly cause saturation of binding sites with transport rates then being dependent only on membrane potential and H^+/H_3O^+ binding.

However, calculating the percentage of the zwitterionic form in the substrate based on the pH of the medium does not take into account the possibility that the environment of the substrate binding site in the transporters may be quite different in terms of hydrophobicity compared with that of the hydrophilic bulk phase. This can dramatically alter the dissociation constants of ionizable groups in the substrate and their ability to hydrogen bond within the binding pocket of the carrier protein (Shan & Herschlag, 1996). Consequently, predictions about the nature of the substrate species that interacts with the substrate binding site may be extremely difficult. Nevertheless, future kinetic studies on mutated or chimeric peptide transporters should provide information about which amino acid residues are involved in substrate and H^+/H_3O^+ binding and the voltage dependence of their interaction.

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Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 249), Projekt B12 to H.D.; S.A. is a recipient of a fellowship from the H. Wilhelm Schaumann Stiftung.

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Received 6 June 1997; accepted 29 July 1997.