PEPT1 as a Paradigm for Membrane Carriers That Mediate Electrogenic Bidirectional Transport of Anionic, Cationic, and Neutral Substrates*

Received for publication, April 30, 2002, and in revised form, June 13, 2002
Published, JBC Papers in Press, June 24, 2002, DOI 10.1074/jbc.M204192200

Gabor Kottra‡, Adelmar Stamfort, and Hannelore Daniel
From the Molecular Nutrition Unit, Technical University of Munich, Hochfeldweg 2, D-85350 Freising-Weihenstephan, Germany

The capability for electrogenic inward transport of substrates that carry different net charge is a phenomenon observed in a variety of membrane-solute transporters but is not yet understood. We employed the two-electrode voltage clamp technique combined with intracellular pH recordings and the giant patch technique to assess the selectivity for bidirectional transport and the underlying stoichiometries in proton to substrate flux coupling for electrogenic transfer of selected anionic, cationic, and neutral dipeptides by the intestinal peptide transporter PEPT1. Anionic dipeptides such as Gly-Asp and Asp-Gly are transported in their neutral and negatively charged forms with high and low affinities, respectively. The positive transport current obtained with monoanionic substrates results from the cotransport of two protons. Cationic dipeptides can be transported in neutral and positively charged form, resulting in an excess transport current as compared with neutral substrates. However, binding and transport of cationic dipeptides shows a pronounced selectivity for the position of charged side chains demonstrating that the binding domain of PEPT1 is asymmetric, both in its inward and outward facing conformation. The simultaneous presence of identically charged substrates on both membrane surfaces generates outward- and, unexpectedly, enhanced inward transport currents probably by increasing the turnover rate.

Most of the rheogenic membrane-solute carriers for cellular uptake of nutrients or xenobiotics carry only a small set of neutral substrates in cotransport with either protons or sodium ions. However, some of the transporters including the proton-coupled intestinal peptide transporter PEPT1 mediate electrogenic substrate influx independent of the net charge of the substrates. In case of PEPT1 there are at least 400 different dipeptides and 8000 different tripeptides that can serve as substrates including all possible mono- or polyvalently charged species. One of the unsolved problems in transport physiology of solutes is how a carrier protein can transport differently charged substrates in the same general transport mode. Several studies have documented that the transport of zwitterionic (at neutral pH) dipeptides by PEPT1 is electrogenic based on a proton to substrate flux coupling ratio of 1:1 (1–4). However, dipeptides carrying net positive or negative charge have also been found to generate transport currents, although cotransport of a negatively charged substrate with protons would be electrically silent when occurring with a fixed 1:1 flux coupling stoichiometry. The observed positive inward current in the case of anionic dipeptides is not unequivocally clear but was suggested to result either from a higher (e.g. 2:1) coupling ratio (4) or from uptake of only the zwitterionic form of the substrate with a 1:1 flux ratio (3) and/or by transport of the anionic substrate and a proton with countertransport of a negatively charged anion (OH\(^-\) or HCO\(_3^-\)) (2).

In case of positively charged dipeptides, transport currents are explained by flux of the cationic substrate together with at least one proton (2, 4) that in turn would lead to a higher current flow than in case of neutral substrates which has, however, never been documented.

Although much attention has been paid to explain the unusually broad substrate specificity of PEPT1, studies regarding the regulation of this transporter by its substrates are very scarce. Only in a few cases have trans-stimulation phenomena been observed, i.e. the presence of transportable substrate on the “trans” side of the cell membrane enhanced the transport of substrate applied to the “cis” side (5–9). These data indicate that substrates bound to the cytosolic binding site of PEPT1 can enhance the inwardly directed transport most likely by an accelerated return of the loaded transporter to the extracellular side (10). The mutual effects of extracellular and intracellular substrates on the transport process have never been systematically studied.

To address the questions of how transport currents of differently charged substrates can be explained and how trans-stimulation can alter transport function, we have characterized the peptide transport function of PEPT1 with the Xenopus expression system and two different electrophysiological methods. By using the giant patch clamp (GPC)\(^1\) technique, we have recently shown that the neutral dipeptide Gly-Gln is transported by PEPT1 not only in the “normal” (inward) but also in the outward direction (i.e. from the cytosol to the extracellular space), even if the apparent substrate binding affinity on the intracellular side was substantially lower. It was also shown that when the dipeptide Gly-Gln was simultaneously present at both membrane surfaces at saturating concentrations and in the absence of a pH gradient, transport is symmetrical and occurs as a linear function of the membrane potential.

Here we analyzed specifically the bidirectional transport of positively and negatively charged dipeptides by employing two

\(^{1}\) The abbreviations used are: GPC, giant patch clamp; TEVC, two-electrode voltage clamp.
pairs of substrates with the same charged amino acid residues in either the amino- or in the carboxyl-terminal position of the dipeptide. Two-electrode voltage clamp recordings (TEVC) and GPC experiments provided evidence that even the position of the charged amino acid side chain in a substrate is affecting the transport process and the associated membrane currents. Simultaneous recordings of transport currents in TEVC and intracellular pH measurements allowed us to calculate proton to charge transfer stoichiometries and the simultaneous application of identical dipeptide substrates to both membrane surfaces in GPC experiments provided trans-stimulation phenomena that shed light onto the carrier cycling and mode of transport.

**MATERIALS AND METHODS**

*Xenopus laevis* oocytes were collected under anesthesia (immersion in a solution of 0.7 g/liter of 3-amino benzoic acid ethyl ester; Sigma) from frogs that were killed with an anesthetic overdose after the final collection. Oocytes were treated with 2.5 mg/ml collagenase for 90 min and were separated manually thereafter. They were incubated in a Barth solution containing (in mM) NaCl 88, KCl 1, MgSO4 0.8, CaCl2 0.4, Ca(NO3)2 0.3, NaHCO3 2.4, HEPES 10 (pH 7.5) at 17 °C overnight. Thereafter stage V/VI oocytes were injected with 25 ng of rabbit PEPT1-cRNA in a 50-nl volume and incubated for 3–6 days at 17 °C.

TEVC and GPC experiments were performed as described previously (11). In short, oocytes were placed in an open chamber and continuously superfused with Barth solution or with solutions containing the substrates to be studied. Oocytes were voltage-clamped at −60 mV using a TEC-05 amplifier (NPI Electronic, Tamm, Germany), and current-voltage (IV) relations were measured immediately before and 20–30 s after substrate application in the potential range of −180 to +90 mV. The current generated by the peptide transport at a given membrane potential was calculated as the difference of the currents measured in the presence and the absence of substrate. Positive currents denote positive charges flowing out of the oocyte.

Patch pipettes of 20–30 μm diameter were prepared from thin-walled borosilicate glass capillaries (MTW-150, WPI, Berlin, Germany) and were filled with a solution containing (in mM) NaCl 10, NaIse 80, MgSO4 1, Ca(NO3)2 1, HEPES 10, titrated to pH 7.5, and connected to an EPC-9 amplifier. After removing of the vitelline layer and gigaseal (1–10 gigaohms) formation pipettes were moved to a perfusion chamber and continuously superfused with the control bath solution composed of (in mM) potassium aspartate 100, KCl 20, MgCl4 4, EGTA 2, HEPES 10 (pH 7.5). During experiments the membrane potential was clamped to −30 mV, and IV relations in the potential range of −120 to +60 mV were measured in the same way as described for TEVC experiments. The data were recorded with the PULSE (HEKA, Lambrecht, Germany) and evaluated with the PATCH (courtesy of Dr. Bernd Letz) program.

Dipeptides (L-isomers) were obtained from Sigma or Bachem, Heidelberg, Germany, and were added to the solutions in concentrations indicated in the text. After addition of dipeptides the pH was readjusted if necessary. The percentage of the zwitterionic form at a given pH was calculated with pH values taken from Ref. 12 or measured at a defined ionic strength matching the perfusate⁵ (Gly-L-Gln, pHK = 8.28; Gly-Asp, pHK = 8.21; Gly-Gln, pHK = 8.45, and pHK = 8.60; Asp-Gly, pHK = 2.10, pHK = 4.53, and pHK = 9.07; Gly-Lys, pHK = 2.90, pHK = 8.20, and pHK = 10.50; Lys-Gly, pHK = 3.00, pHK = 8.10, and pHK = 10.70).

The transport parameters Kᵣ (mM) and Iᵢₘₐₓ (nA or pA) were calculated from least square fits to the Michaelis-Menten equation. To be able to perform kinetic analysis and to determine validly Iᵢₘₐₓ values that depend on the expression level of the transporter, all substrate concentrations and pH effects were analyzed in the same oocyte. This required a reduction of the number of data points for analysis from 3 to 4 in TEVC and to 5 in GPC experiments. Substrate concentrations were 0.5, 2, and 5 mM for Lys-Gly (pH 7.5 and 8.5), Gly-Lys (pH 7.5), Gly-Asp and Asp-Gly (pH 5.5), whereas 2.5, 5, and 10 mM were used for Gly-Lys (pH 8.5) and Asp-Gly (pH 7.5), and 2, 5, 10, and 20 mM were applied to calculate the kinetic parameters of Gly-Asp at pH 7.5. In GPC experiments, the concentrations were 1, 2.5, 5, 10, and 20 mM for all substrates tested.

To determine charge to proton flux ratios of different dipeptides, TEVC measurements were combined with intracellular pH measure-ments. pH microelectrodes were made from borosilicate capillaries that were pulled to about 10 μm tip diameter. Electrodes were silanized as described before (13), and the tip was filled with Fluka hydrogen ionophore I mixture B (catalog number 95293) overnight. Electrodes were backfilled prior to use with a solution containing (in mM) KH2PO4 40, NaOH 23, NaCl 15, pH 7.0. pH electrodes were connected to an FD223 amplifier (WPI, Berlin, Germany). The reference channel of the amplifier was connected to the voltage output signal of the TEC-05 amplifier. The slope of the pH decline between 30 and 150 s was taken as a measure of acidification and was related to the charge transfer that was calculated as the integral of substrate-evoked current flow during the same period. Because intracellular pH did often not completely recover during a 5–10-min washout period after the first substance application, to avoid systematic errors the application sequence of dipeptides was varied.

Data are given as the mean ± S.E. of n experiments. Statistically significant differences (p < 0.05) were determined with the Student’s t test for paired or non-paired data as appropriate.

**RESULTS**

**Inward and Outward Transport of Charged Dipeptides**—Fig. 1 shows transport current traces of the selected zwitterionic, monocationic, or monoanionic dipeptides (10 mM each) at two pH values in the same oocyte. At pH 7.5 about 86% of Gly-Gln and ~17% of Lys-Gly but only less than 0.1% of Gly-Asp were present in the electrically neutral, zwitterionic form. Thus, over 99.9% of Gly-Asp was negatively charged, whereas about 83% of Lys-Gly was positively charged. Fig. 1 (left side) shows that at pH 7.5 Lys-Gly induced considerably higher currents than Gly-Gln, whereas Gly-Asp evoked less current than the neutral Gly-Gln. Because Gly-Gln is known to have a high affinity to PEPT1 (Kᵣ < 1 mM), the transporter can be expected to be saturated at 10 mM substrate concentration. The higher current in case of Lys-Gly can either result from a higher turnover rate of the transporter or, more probably, from the cotransport of charged substrate molecules with protons resulting in a charge to substrate stoichiometry of >1. On the other hand, the transport of a negatively charged Gly-Asp molecule together with one proton would be electrically silent. Thus, the inward current recorded at pH 7.5 denotes either the transport of the neutral form of the peptide or an extremely high proton to transport of the negatively charged form together with two protons. As shown in Fig. 1, a reduction of pH from 7.5 to 5.5, which increases the percentage of the neutral form of Gly-Asp to ~8%, increased Gly-Asp-induced currents to the same level as those of Gly-Gln that remained unaffected by the pH change. This observation strongly suggests that PEPT1 transports
preferentially, but not exclusively, the zwitterionic form of Gly-Asp.

To obtain more detailed information on the role of substrate charge in relation to transport kinetics ($K_m$ and $I_{max}$) and their dependence on pH and membrane potential, we analyzed pairs of dipeptides containing a basic or an acidic amino acid side chain in either the carboxyl- or in the amino-terminal position (Lys-Gly, Gly-Lys, Gly-Asp, and Asp-Gly). TEVC measurements provided data for the inward transport direction, and similar data were obtained from inside-out giant patch clamp experiments for the outward transport direction. These data were compared with the respective parameters of the zwitterionic dipeptide Gly-Gln as reported previously (11) and obtained under identical experimental conditions. Representative kinetics of current responses as a function of substrate concentrations are shown in Fig. 2A for TEVC analysis of Gly-Asp transport at two membrane potentials and for GPC analysis of Lys-Gly transport in Fig. 2B. Summarized results are provided in Table I and in Fig. 3 for Lys-Gly and in Fig. 4 for Gly-Asp, respectively. All kinetic constants have been calculated only for limited potential ranges (TEVC, -160 to -60 mV; GPC, -20 to +60 mV), because outside of these limits transport rates are generally too low to deliver valuable transport data.

**Cationic Dipeptides**—The cationic dipeptides Lys-Gly and Gly-Lys were progressively deprotonated when pH was elevated from 7.5 to 8.5, and the percentage of the positively charged species decreased from 83 to 33%. The simultaneous changes in substrate affinity were moderate, especially in case of Gly-Lys. However, at pH 7.5 the $K_m$ value for Gly-Lys was about 6-times higher than for Lys-Gly. This difference in $K_m$ value would be expected, when both the charged and uncharged form of Gly-Lys but only the neutral form of Gly-Lys were transported by PEPT1. In fact, $I_{max}$ was higher than that of Gly-Gln only in case of Lys-Gly but not in case of Gly-Lys. This strongly suggested that Lys-Gly but not Gly-Lys was transported both in its neutral as well as positively charged form. The additional current evoked by Lys-Gly at pH 7.5, when compared with that of Gly-Gln, was almost as high (+68%) as the increased percentage of Lys-Gly in its positively charged form (83%). When pH was increased to 8.5, $I_{max}$ induced by Lys-Gly decreased to 107% of the respective Gly-Gln current, and this decrease, although slightly more than expected, appears because of the decline in the percentage of the positively charged species.

In summary, we demonstrate here for the first time that cationic dipeptides possess different transport characteristics, depending on the location of the charged side chain within the substrate molecule. Except from differences in affinity, Lys-Gly but not Gly-Lys can be transported both in its charged and non-charged form.

Outward transport currents in GPC studies could only be measured for Lys-Gly, whereas Gly-Lys did not induce any currents. As for other dipeptides, the binding affinities on the inner membrane surface were almost 15 times lower than the corresponding affinity for binding and transport of the same substrates in the inward-facing direction (for both pH values at +60 mV). $I_{max}$ in case of Lys-Gly reached 182 (at pH 7.5) and 141% (at pH 8.5) of the currents produced by neutral Gly-Gln. These values correspond well to expected additional charge transfer when positively charged and neutral forms are transported simultaneously with the same affinity.

With the information that the kinetics of PEPT1 was dependent on the membrane potential, we determined the membrane potential dependence of the kinetic parameters for Lys-Gly transport (Fig. 3, A and B). $I_{max}$ as a function of membrane potential was for both transport directions nearly independent of the actual pH (7.5 or 8.5). This suggests that the charge of the substrate was more important in affecting maximal transport currents than the difference in the transmembrane pH gradient of one unit. $K_m$ values increased toward 0 mV membrane potential independently of pH and transport direction. This observation is in support of the assumption that an increasing negative potential on the trans side of the cell membrane “attracts” the positively charged substrate molecules toward the binding site on the transporter.

The marked differences obtained in transport currents evoked by Lys-Gly and Gly-Lys have prompted us to investigate whether similar differences can be observed with other dipeptides containing the basic amino acid arginine in either the carboxyl- or in the amino-terminal position. Like Lys-Gly, about 80% of Arg-Gly and Gly-Arg are positively charged at pH 7.5. In TEVC experiments we found a moderate difference in...
the substrate affinities ($K_m$) of Arg-Gly, 1.06 ± 0.06 mM; $K_m$ of Gly-Arg, 1.93 ± 0.09 mM at pH 7.5 and -60 mV), but $I_{\text{max}}$ determined for Arg-Gly was higher by 17 ± 2% than $I_{\text{max}}$ calculated for Gly-Gln. The maximal currents of Gly-Gln and Gly-Arg were not significantly different. So, Arg-Gly-like Lys-Gly produces higher currents, although less pronounced, which suggests that it also may be transported in part in positively charged form. Our results obtained for the four cationic dipep-

**TABLE I**

Kinetic transport parameters of uncharged and charged dipeptides in the inward and outward direction

| Substrate | pH  | Neutr | $I_{\text{max}}$ % | $K_m$ (mM) | $K_m$ (mV) |
|-----------|-----|-------|-------------------|------------|------------|
| Gly-Gln   | 7.5 | 86    | 100 ± 0           | 0.42 ± 0.02| 0.70 ± 0.04|
| Gly-Asp   | 5.5 | 8     | 110 ± 6           | 0.78 ± 0.16| 0.33 ± 0.08|
| Asp-Gly   | 5.5 | 10    | 84 ± 3            | 0.95 ± 0.09| 0.45 ± 0.04|
| Gly-Lys   | 7.5 | 17    | 100 ± 2           | 3.16 ± 0.55| 4.59 ± 0.30|
| Lys-Gly   | 7.5 | 17    | 168 ± 8           | 0.36 ± 0.07| 0.78 ± 0.11|
| Gly-Gln   | 8.5 | 67    | 108 ± 6           | 0.93 ± 0.04| 1.78 ± 0.15|

For $I_{\text{max}}$, standard errors were omitted to increase transparency. Note the different scales for $K_m$.
tides demonstrate that when the positive charge is located in the carboxyl-terminal position of the substrate, its accommodation in the substrate binding domain is not possible. When the side chain charge is located in the amino terminus, the charged substrate is bound and carries the additional current during substrate translocation. This strongly suggests that the substrate binding domains in PEPT1 that accommodate the various side chains are asymmetric.

**Anionic Dipeptides**—The percentage of the neutral form of the anionic dipeptides Gly-Asp and Asp-Gly increases from <0.1 to 8–10% upon a pH reduction from 7.5 to 5.5. This led to a marked increase in binding affinity to PEPT1 (Gly-Asp, ~39-fold; Asp-Gly, ~6.8-fold), whereas the simultaneous changes in \[I_{\text{max}}\] were only moderate (Table I). For the reverse transport direction, Gly-Asp showed a similar increase in binding affinity upon pH reduction, although its \[K_m\] value remained even at pH 5.5, almost 10-fold higher than for the inward transport direction. Asp-Gly when added to the cytosolic surface was not transported in the outward direction suggesting an extremely low affinity.

The potential dependence of the kinetic parameters for Gly-Asp transport is shown in Fig. 4, A and B. The potential dependence of \[I_{\text{max}}\] was for both directions less pronounced at a pH of 5.5 than at a pH of 7.5 suggesting that the transmembrane pH gradient of two pH units in the former case contributed to the transport when membrane potentials change toward 0 mV. \[K_m\] at pH 5.5 was potential-independent for the outward direction, and only slightly potential-dependent for the inward direction. In contrast, \[K_m\] at pH 7.5 increased for both directions with membrane hyperpolarization.

**Analysis of Transport by Combined Voltage Clamp and pH Measurements**—The comparative analysis of \[I_{\text{max}}\] values for Gly-Gln and Lys-Gly transport as well as the observed pH dependence suggested that Lys-Gly is transported both in its positively charged and electrically neutral form with similar affinities. Based on this assumption, a proton to charge stoichiometry of about 1.8 for Lys-Gly at pH 7.5 had to be assumed. To analyze the corresponding changes in intracellular pH, we measured \[pH_{\text{in}}\] with pH-selective microelectrodes under voltage clamp conditions in response to nearly saturating concentrations of Lys-Gly and Gly-Gln. If the excess current evoked by Lys-Gly indeed resulted from the transport of positively charged substrate molecules and not from a higher proton to substrate stoichiometric ratio, then the pH change/current ratio should be lower for Lys-Gly than for Gly-Gln. Fig. 5 shows that this was indeed the case. Although the membrane current induced by the transport of Lys-Gly was considerably higher than that induced by Gly-Gln, the rate of intracellular acidification was almost identical. In 10 independent experiments the mean ratio of currents evoked by Lys-Gly versus that of Gly-Gln was 1.64 ± 0.11, whereas the mean ratio of pH changes based on the slope of intracellular acidification was 0.74 ± 0.05. The pH/current ratio for Lys-Gly was only 47 ± 4% of the respective value for Gly-Gln, confirming that at identical transport currents Lys-Gly induced less acidification than Gly-Gln. The data of this series of experiments for the various substrates are summarized in Table II. In contrast to Lys-Gly, Gly-Lys is transported mainly in its neutral form, and currents identical with those evoked by Gly-Gln lead to comparable pH changes. When the cationic dipeptides Lys-Gly and Gly-Lys were applied at an extracellular pH of 8.5, and thus were present in the bath mainly (around 67%) in their neutral form, their uptake into the now more acidic (~7.4) intracellular compartment yielded greatly reduced cytosolic pH changes as the substrates by themselves obviously increase intracellular buffering capacity.

To address the question of whether Gly-Asp at pH 7.5 is transported with a 2:1 (or larger) proton to substrate stoichiometry, as suggested by the TEVC experiments, we compared transport currents and corresponding \[pH_{\text{in}}\] changes of Gly-Gln and Gly-Asp. Both substrates were applied at a concentration of 20 mM and at pH values of 7.5 and 5.5. As shown in Table II, the proton to charge stoichiometric ratios for Gly-Asp versus Gly-Gln transport were 166 and 170% at pH 7.5 and 5.5, suggesting that a considerable part of the current induced by Gly-Asp resulted from the transport of the anionic species together with more than one proton. Because the percentage of the neutral form of this dipeptide increases upon pH reduction.

### Table II

**Combined TEVC and pH measurements in Xenopus oocytes**

All substrates were applied at 20 mM concentration. All data are relative values calculated as quotient of the values measured with the substance and at the pH given in the left column divided by the corresponding value measured with Gly-Gln at pH 7.5 in the same oocyte.

| Substrate and pH | Current \[nA\] | \[pH\] change | \[pH\]/current |
|-----------------|----------------|----------------|----------------|
| Lys-Gly, 7.5    | 1.64 ± 0.11    | 0.74 ± 0.05    | 0.47 ± 0.04 (\(n = 10\)) |
| Lys-Gly, 8.5    | 1.06 ± 0.02    | −0.12 ± 0.06   | −0.12 ± 0.06 (\(n = 4\)) |
| Gly-Lys, 7.5    | 0.98 ± 0.07    | 0.81 ± 0.08    | 0.81 ± 0.04 (\(n = 10\)) |
| Gly-Lys, 8.5    | 0.61 ± 0.03    | 0.08 ± 0.10    | 0.11 ± 0.17 (\(n = 4\)) |
| Gly-Asp, 7.5    | 0.47 ± 0.05    | 0.77 ± 0.12    | 1.66 ± 0.22 (\(n = 8\)) |
| Gly-Asp, 5.5    | 0.69 ± 0.12    | 1.26 ± 0.39    | 1.70 ± 0.20 (\(n = 5\)) |
| Asp-Gly, 7.5    | 0.72 ± 0.05    | 0.75 ± 0.12    | 1.05 ± 0.16 (\(n = 9\)) |
| Asp-Gly, 5.5    | 0.53 ± 0.02    | 1.01 ± 0.14    | 1.88 ± 0.22 (\(n = 7\)) |
from 7.5 to 5.5 only by ~8% (see Table I), no major buffering effect on intracellular pH was expected and observed. Similar results were also obtained with Asp-Gly at pH 5.5, whereas the calculated transport stoichiometric ratio at pH 7.5 was, unexpectedly, not significantly different from 100%.

**Inward and Outward Transport of Charged Dipeptides Assessed in Patch Clamp Experiments**—We previously demonstrated that inward currents generated by PEPT1 in inside-out giant membrane patches can be blocked completely by applying the inhibitor glibenclamide to the cytosolic surface and that this allows us to record a zero-line in the presence of substrate on the extracellular side (11). It has also been shown that in the presence of a saturating Gly-Gln concentration on both membrane surfaces, the transporter showed symmetry, and transport direction and current were determined solely by the membrane potential. The same protocol was used here to investigate the effects of charged dipeptides on transport characteristics when provided at both membrane sites. For these studies we selected the dipeptides Lys-Gly and Gly-Asp which were shown to be transported in both directions (see Table I).

Fig. 6A shows the results obtained with Lys-Gly. At a membrane potential of +60 mV and in the absence of cytosolic substrate, the current was inwardly directed, but the addition of substrate to the inner side generated a concentration-dependent outward current. Surprisingly, addition of Lys-Gly to the cytosolic surface increased the inward current, although reduced currents by a reduction of the driving force were expected. The increase in both the outward and inward current components resulted in a rotation of the I-V relationship around -10 pA and a membrane potential of about -10 mV (Fig. 6A). Simultaneously, increasing substrate concentrations shifted the zero-current potential of the I-V relationship toward 0 mV. This shift of the zero-current potentials was used to calculate the charge to substrate stoichiometry of the transport of Lys-Gly. Linear regression analysis of the reversal potential versus log concentration resulted in an estimated shift of 26.9 mV/concentration decade. From this value, a stoichiometric ratio of 2.18 was calculated, which is close to that observed in the TEVC studies for inward transport. However, the dose-dependent increase of the inward current despite the declining substrate gradient reveals a trans-stimulation phenomenon not observed with a zwitterionic substrate.

To calculate the stoichiometry ratio of Gly-Asp transport at pH 7.5, similar experiments were made at a substrate concentration of 20 mM provided on the outside and 0, 2, 10, or 20 mM on the cytosolic side. Due to the low affinity of this dipeptide for binding on the inside (see Table I), only few recordings yielded currents that could be analyzed. As shown in Fig. 6B, Gly-Asp-mediated currents also showed potential dependence with a rotation around an interception point where current remains nearly constant, in this case at -60 mV. At depolarizing membrane potentials and in the absence of cytosolic dipeptide, the current was inwardly directed, but the addition of not more than 2 mM Gly-Asp to the cytosolic bath already induced a small outward component at +60 mV. The total shift of the reversal potential upon the increase of cytosolic substrate concentration was 50 mV, suggesting a stoichiometric substrate to charge ratio only slightly above 1.

The experiments described here also allowed us to calculate the apparent $K_m$ values for binding of charged dipeptides on the cytosolic surface with the simultaneous presence of identical substrates on the extracellular side. The apparent $K_m$ of Lys-Gly was 15.2 ± 2.6 mM in the presence of extracellular substrate (n = 3) as compared with 11.9 ± 2.5 (see Table I) and that of Gly-Asp was 17.3 ± 5.5 mM (n = 3) as compared with 14.3 ± 3.6 in the absence of extracellular substrate. So substrate affinities for binding to the substrate binding domain of PEPT1 in its cytosol facing conformation are not at all affected by substrate present in the outside.

**DISCUSSION**

The electrogenic transport not only of neutral but also of charged dipeptides by the intestinal transporter PEPT1 addresses one of the unsolved problems in transport physiology, and that is how the protein can handle differently charged substrates in the same transport mode. We have determined the transport characteristics of glycyl-dipeptides carrying non-charged or charged side chains by measuring transport currents under voltage clamp conditions in oocytes simultaneously with changes in pH$_m$ and by applying the giant patch technique. From analysis of transport currents and intracellular pH measurements, we conclude (see Fig. 7) the following: (a) the cationic dipeptide Lys-Gly is transported in both the zwitterionic and positively charged form, whereas Gly-Lys is transported by PEPT1 only in its neutral form; (b) the anionic dipeptides Gly-Asp and Asp-Gly are transported in uncharged...
pH-dependent higher charge transfer for Lys-Gly and a higher terminal position. Thus, at pH 7.5 nearly 100% of Lys-Gly but only neutral ones in the carboxyl-terminal position. PEPT1 can accommodate neutral or positively charged side chains in the inward direction. The substrate binding pockets of the cytosolic solution the additional proton is dissipating again resulting in and are translocated via PEPT1 in their neutral form. In the bulk membrane presumably by the acidity resulting from proton backflux Asp-Gly and Gly-Asp are protonated in the very proximity of the cell membrane presumably by the acidity resulting from proton backflux and are translocated via PEPT1 in their neutral form. In the bulk cytosolic solution the additional proton is dissipating again resulting in an enhanced acidification on the cytosolic surface.

form, but a minute fraction of charged form can be transiently protonated in the very proximity of the membrane and is thus translocated virtually as charged species together with two protons; and (c) Lys-Gly and Gly-Asp but not Gly-Lys and Asp-Gly can bind to PEPT1 on the cytosolic site and are transported in the outward direction.

From our analysis we conclude that the substrate binding pocket of PEPT1 is asymmetric, both in its inward and outward facing conformation as well as with respect to the accommodation of the different side chains in substrates. Apparent substrate affinities of all dipeptides tested are generally much lower on the inside than the outside under otherwise identical conditions and show a pronounced dependence for the amino- or carboxyl-terminal location of charged in a substrate. The simultaneous presence of charged substrates on the extracellular and cytosolic membrane side amplifies inward and outward currents in an unexpected manner depending on transmembrane potential and the net charge of the substrate.

Stoichiometry of Flux Coupling—Although previous studies (2, 4) already suggested that PEPT1 can transport dipeptides with a charge to substrate stoichiometry ratio other than 1:1, we unequivocally demonstrated here that even dipeptides with the same chemical composition and carrying the same charge show different transport characteristics. In the case of Lys-Gly, both I_max calculations and simultaneous current and pH measurements under voltage clamp conditions led us conclude that this substrate and to a lesser extent also Arg-Gly are transported in neutral as well as in positively charged form and therefore generate higher transport currents than zwitterionic substrates. This was not the case for the reversed substrates Gly-Lys and Gly-Arg that possess essentially the same dissociation constants for the ionized groups but in a different conformational position. Based on our data only 17% (about 1/6th) of the Gly-Lys molecules (those present in neutral form) are recognized by the transporter in contrast to 100% of the Lys-Gly molecules, and correspondingly the K_m values at pH 7.5 vary about 6-fold. The higher K_m value for Gly-Lys than for Lys-Gly (2.2 versus 0.91 mM) has also been found at an extracellular pH 6 in a previous study (14). PEPT1 therefore binds cationic glycyl-dipeptides (at least those with lysine residues) much better when the extra charge is in the amino-terminal position. It is interesting to note that the modification of the e-amino group of an amino-terminal lysine residue in a dipeptide with either a Z-group or a Z(NO_2)-group renders such a compound into an effective inhibitor of PEPT1, whereas identical modifications in carboxyl-terminal lysine residues retained the compounds capable of transport (15). This all strongly argues for asymmetry in the binding pockets that accommodate amino- or carboxyl-terminal side chains, but this asymmetry not only affects substrate affinity but also the charge transfer ratio in a transport cycle. New peptide transport inhibitors that block the transport cycle of the closely related PEPT2 protein also suggest distinctly different pockets that accommodate the side chains and that may contain different residues that interfere with the charged side chains in substrates (16).

Published transport data on monoanionic dipeptides showed electrogenicity, but there is no agreement on how this positive inward current is generated. It could result (a) from the co-transport of one substrate molecule together with two protons (3, 4), or (b) from the cotransport of one proton per substrate molecule and a simultaneous countertransport of one negatively charged counterion such as OH^- or HCO_3^- (2). An alternative explanation could be the preferential transport of only the zwitterionic form of the substrate with one proton (17, 18) with unknown changes in the pK_a values of the ionized groups in the hydrophobic membrane environment. Because current measurements alone do not allow the distinction between these possibilities, we have simultaneously measured transport currents and acidification rates of the cytosolic compartment under voltage clamp conditions. At a pH_out of 5.5 and at equal currents, transport of Gly-Asp is accompanied by ~1.7 times faster intracellular acidification rate than in case of Gly-Gln, suggesting that the majority of Gly-Asp is transported with two protons from which one could be carried by the protonated side chain carboxyl group. In addition, lowering pH titrates more substrate into its neutral form, and this form shows increased binding affinity to PEPT1 on both membrane faces. When the zwitterionic substrate form is bound and transported in the inward direction, the higher intracellular pH causes deprotonation of Gly-Asp with the delivery of the additional proton and turns the dipeptide into a charged, but now low affinity type substrate. A possible countertransport of an anion such as OH^- or HCO_3^- which would acidify the cytosol as suggested previously (2) would require a further binding pocket for such an anion on the “trans”-side (the cytosolic side) of PEPT1. This anion binding pocket would be needed and occupied only when anionic dipeptides are transported, and this seems most unlikely.

![Fig. 7. A scheme for the transport of charged dipeptides by PEPT1 in the inward direction.](image-url)
In the case of the tested anionic dipeptides, the shift of the acidic side chain from the amino- to the carboxyl-terminal half changed binding affinities only modestly. The $K_m$ values were very similar but increased for both substrates dramatically at low pH (pH 5.5) when the percentage of the zwitterionic species increased from essentially 0 to 8–10% of total substrate present.

Michaelis-Menten kinetic parameters open the possibility to compare not only substrate affinities but also the $I_{\text{max}}$ currents which, on the other hand, allow some insights into the mode of operation of the transporter. Pre-steady state kinetics of PEPT1 (19) were consistent with an ordered, simultaneous transport model in which protons bind first to the transporter followed by substrate binding and translocation. Theoretical analysis predicts in such a model that $I_{\text{max}}$ currents at saturating substrate concentration become independent of the concentration of the cotransported ion ($H^+$) (20). Our present data do not fully support the model proposed by Mackenzie and co-workers (19) because the $I_{\text{max}}$ values do in some cases show a dependence on the pH (i.e. the concentration of the cotransported ion) for both the inward and outward direction of transport. The largest deviations could be observed with Gly-Lys in the inward and Gly-Asp in the outward direction. This might be explained by pH-dependent changes in substrate charge but also by pH-dependent alterations of the transporter protein and its conformation. However, the exact determination of $I_{\text{max}}$ here is limited by the low affinities, and a more detailed analysis is required to judge whether the proposed model is valid in all cases.

Trans-stimulation—We have also analyzed how transport rate of a given substrate is altered in the presence of substrate on the cytosolic side which resembles a physiological condition. Only very few studies using oocytes, renal or intestinal brush-border membrane vesicles, or epithelial cell in culture that express PEPT1 have analyzed trans-stimulation events in peptide transport (5, 6, 8) or PEPT2 (5, 7, 9). An acceleration of the transporter-mediated influx of peptide substrates was occasionally observed when cells or vesicles were preloaded with dipeptides or peptide-like drugs in tracer flux studies. These trans-stimulation phenomena are in accordance with a model proposed in which the turnover rate of the carrier is determined by the return of the unloaded protein to the outer surface and in which the loaded transporter could cycle faster back to the external membrane site (10).

GPC studies as used in the present study offer important advantages in analyzing such mutual cis-trans effects on substrate translocation. In GPC experiments, the substrates and their concentrations on both membrane surfaces can be freely selected, and by recording I-V relationships, the potential dependence of trans-stimulation processes can be measured. Moreover, alterations of the membrane potential on inward and outward transport processes can be investigated independently. In the case of PEPT1, at high negative membrane potentials (−120 mV), inwardly directed transport dominates, whereas at +60 mV the inward current is reduced and current flows into the reverse direction.

Our GPC experiments performed in the absence of cytosolic substrates show unidirectionality of transport, because in this model system, transport was not able to build up significant substrate concentrations on the trans-side. Addition of substrate to the cytosolic side reduces the transmembrane substrate gradient and results in a bidirectional transport (of the same substrate) probably at an increased turnover rate (see above). When the flux coupling ratio for substrate to cotransported proton is equal in both directions, the net inward current will decrease by outwardly directed transport despite the increased turnover rate. This was indeed observed in our previous experiments with Gly-Gln (11). In the present study we observed important differences in transport behavior between uncharged and charged dipeptides. Instead of reducing the steady state inward transport currents of Lys-Gly and Gly-Asp by cytosolic substrate addition, currents increased in a concentration-dependent fashion when the same substrate species was present inside. Now, this would be a strong indication of a trans-stimulation event, but it also suggests that the stoichiometric ratios in the inward and outward transport direction may not be equal. At high inside negative membrane potentials more positive charges per transported substrate molecule must pass the membrane in the inward direction than in the opposite direction for increased influx currents.

For Lys-Gly, a possible explanation might be that at physiological pH (pH 7.5 on both membrane sides) about 83% of all substrate molecules are protonated, i.e. positively charged with the remainder in zwitterionic form. A negative potential on the trans side of the membrane evokes an electrophoretic movement and attracts the positively charged molecules toward the membrane resulting in a higher local concentration in the vicinity of the membrane. The positively charged species then preferentially occupies the binding site of PEPT1. On the opposite membrane surface the concentration of positively charged molecules decreases in relation to the zwitterionic species leading to a lower charge to substrate flow coupling ratio.

The same explanation may not be valid for Gly-Asp that is present at pH 7.5 almost exclusively in its negatively charged form. Because two cotransported protons per Gly-Asp molecule are required to generate a positive current flow, increased inward currents in the presence of Gly-Asp on the inside can only be explained if transport into the outward direction occurs with less charge transfer, i.e. with none or only one instead of two protons. However, an electrically silent transport of this substrate could not be observed in our experiments, and therefore the possibility that other cotransport stoichiometries, such as 3 protons in the inward and 2 protons in the outward direction, have to be taken into account. The concentration of protons present in the vicinity of the membrane and thus the local pH might be influenced by the electrochemical forces imposed by the actual membrane potential, and these differences might then influence the protonation/deprotonation state of the substrate molecules when entering the substrate binding pocket of the transporter. However, a proton to substrate stoichiometry of 3:1 has been proposed for influx of dipeptides by the peptide transporter PEPT2 but not yet for PEPT1 (21).

In summary, by combining classical two electrode voltage clamp studies with simultaneous intracellular pH recordings and the giant patch clamp technique, we provide evidence for asymmetry in the substrate binding domain of PEPT1 that can transport positively charged dipeptides only when the charged side chain is provided in an amino-terminal position and which then causes increased transport currents at the same turnover rate. The asymmetry in the substrate binding domain is maintained when the transporter faces the cytosol which then discriminates substrates even more pronounced and allows only certain dipeptides to be transported in the outward direction. Different coupling ratios for proton to substrate fluxes explain the electrogenic character in inward transport of zwitterionic and monovalently charged substrates at a given carrier turnover rate.

Acknowledgment—We acknowledge the expert technical assistance of Rainer Reichlmeir.
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