Mechanisms of the Human Intestinal H⁺-coupled Oligopeptide Transporter hPEPT1*

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The hPEPT1 cDNA cloned from human intestine (Liang, R., Fei, Y.-J., Prasad, P. D., Ramamoorthy, S., Han, H., Yang-Feng, T.-L., Hediger, M. A., Ganapathy, V., and Leibach, F. H. (1995) J. Biol. Chem. 270, 6456–6463) encodes a H⁺/oligopeptide cotransporter. Using two-microelectrode voltage-clamp in Xenopus oocytes expressing hPEPT1, we have investigated the transport mechanisms of hPEPT1 with regard to voltage dependence, steady-state kinetics, and transient charge movements. The currents evoked by 20 mM glycyl-sarcosine (Gly-Sar) at pH 5.0 were dependent upon membrane potential (Vm) between −150 mV and +50 mV. Gly-Sar-evoked currents increased hyperbolically with increasing extracellular [H⁺], with Hill coefficient ~1, and the apparent affinity constant (K0.5) for H⁺ was in the range of 0.05–1 μM. K0.5 for Gly-Sar (K0.5) was dependent upon Vm and pH; at −50 mV, K0.5 was minimal (~0.7 mM) at pH 6.0. Following step-changes in Vm, in the absence of Gly-Sar, hPEPT1 exhibited H⁺-dependent transient currents with characteristics similar to those of Na⁺/coupled transporters. These charge movements (which relaxed with time constants of 2–10 ms) were fitted to Boltzmann relations with maximal charge (Qmax) of up to 12 nC; the apparent valence was determined to be ~1. Qmax is an index of the level of transporter expression which for hPEPT1 was in the order of 10³/oocyte. In general our data are consistent with an ordered, simultaneous transport model for hPEPT1 in which H⁺ binds first.

In the mammalian intestine, absorption of oligopeptides is mediated by one or more proton-coupled membrane transporters (reviewed in Refs. 1 and 2). The sequential actions of the Na⁺/K⁺-ATPase pump and the Na⁺/H⁺ exchanger in the small intestine create an acidic extracellular microclimate (pH ~ 6.0) generating an inward H⁺ electrochemical gradient sufficient to drive the tertiary active transport of oligopeptides.

Liang et al. (4) cloned and expressed a human intestinal cDNA encoding a H⁺-dependent oligopeptide transporter (hPEPT1).1 hPEPT1 accepts a broad range of substrates including dipeptides and tripeptides, but not free amino acids; hPEPT1 also accepts as substrates certain pharmacologically active compounds such as the tripeptide-like β-lactam antibiotics and the antineoplastic agent bestatin. hPEPT1 is homologous (81% amino acid sequence identity (4)) with the rabbit H⁺/oligopeptide cotransporter (rPEPT1): transport is electrogenic, coupled to an influx of H⁺, and independent of Na⁺, K⁺, and Cl⁻ (5, 6).

We have investigated the mechanisms of H⁺/oligopeptide cotransport mediated by the human intestinal PEPT1 transporter and describe the biophysical and kinetic characterization of hPEPT1 using two-microelectrode voltage-clamp in cRNA-injected oocytes, with the non-hydrolyzable dipeptide glycyl-sarcosine (Gly-Sar) as the characterizing substrate.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions—All chemicals were from Sigma except where specified; restriction enzymes were from Stratagene (La Jolla, CA) and Promega (Madison, WI). For oocyte injections, RNA was suspended in vehicle containing 1 mM Na₂EDTA, 10 mM HEPES (pH 7.0 with KOH). Oocytes were superfused with experimental medium of composition: 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (buffered to a range of pH values between 5.0 and 7.5 using Tris/HCl); NaCl was replaced by choline chloride (ChCl) for Na⁺-free solutions.

Preparation of Oocytes—Oocytes, isolated from Xenopus laevis (Nasco, Fort Atkinson, WI), were defolliculated with collagenase B (Boehringer Mannheim), followed by K₂HPO₄ (7). Defolliculated mature (stage V-VI) oocytes were selected and maintained at 18 °C in modified Barth’s medium (7) with 10 mg/liter−1 gentamicin sulfate.

Subcloning of the Full-length hPEPT1 cDNA Sequence—The cloning of a 2.2-kb cDNA fragment containing the coding sequence for hPEPT1 has been described previously (4); however, this truncated cDNA lacked a 3¢ poly(A) tail. Using the same strategy (4) we isolated a second cDNA fragment (of 2.5 kb) containing the 3¢-translated region and poly(A) tail but which lacked a 5¢-coding region of 0.6 kb. Both cDNA fragments were subcloned into the Bluescript II SK− (−) phagemid (Stratagene) to construct plasmids pHPEPT1A (original 2.2-kb fragment) and pHPEPT1B (new 2.5-kb fragment). Sequencing revealed that both cDNA fragments were in the same orientation within the vector (downstream from the T7 promoter) and an overlapping region between pHPEPT1A and pHPEPT1B containing a unique restriction site (NsiI) was identified. Double digestion of pHPEPT1B with NsiI (in the insert) and Xbal (in the multiple cloning sequence) yielded a 1.8-kb cDNA fragment containing the entire 3¢-translated region and part of the coding region. This 1.8-kb fragment was gel-purified using the Gene clean® kit (BIO 101, La Jolla, CA) and subcloned into the pHPEPT1A plasmid, after double digestion of pHPEPT1A (with NsiI and Xbal) and removal of a ~1.0-kb digest fragment by gel purification, to generate a new plasmid pHPEPT1 containing the 3.1-kb full-length cDNA. DH5α competent cells (Life Technologies, Inc., Grand Island, NY) were transformed with pHPEPT1 and plasmid DNA was prepared using the CSCI equilibrium ultracentrifugation method. Sequencing (8) was performed on the 3.1-kb construct and the sequence information submitted to
GenBank (accession number U21936). cRNA Synthesis—Plasmid pHPEPT1 was linearized with BamHI and transcribed in vitro using T7 RNA polymerase (Stratagene), RNase inhibitor, and RNA cap analog, m'G(5')ppp(5')G (Pharmacia, Piscataway, NJ), or the Ambion MEGAscript kit with cap analog and T7 RNA polymerase (Ambion, Austin, TX). The rabbit clone (pRPEPT1) contained the PEP1T-cDNA (5) in the pSPORT vector under the control of the T7 promoter. The plasmid was linearized by BamHI digestion and rPPEPT1 cRNA synthesized using the Ambion kit. The cRNAs were each phenol-chloroform extracted and ethanol precipitated; concentration was determined by UV spectrophotometry and the integrity of the RNA verified by denaturing 1% formaldehyde-agarose gel electrophoresis and visualization using ethidium bromide fluorescence.

Expression of hPEPT1 and rPEPT1 in Oocytes—Oocytes were injected 1 day after isolation with ~50 ng of hPEPT1 cRNA or rPEPT1 cRNA and incubated at 18°C.

Electrophysiology Methods—A two-microelectrode voltage-clamp system (9) was used to measure presteady-state and steady-state currents associated with hPEPT1 and rPEPT1 expressed in oocytes, 5–7 days after cRNA injection. Oocytes were superfused at 20–22°C and held at −50 mV (V_m); step changes in membrane potential (V_m) were applied, each for a duration of 100 ms (+50 to −150 mV in 20-mV increments), first in Na⁺ medium at pH 7.5–5.0, then after superfusing 1–2 min with glycyly-sarcosine (Gly-Sar). The currents were averaged over three sweeps and low-pass filtered at 500 Hz by an eight-pole Bessel filter. Test solutions were always washed out by superfusing the oocyte with substrate-free, ChoCl medium (pH 7.5). Steady-state data were fitted to Equation 1, for which substrate-free, ChoCl medium (pH 7.5). Steady-state data were fitted to Equation 1, for which  

\[ I = \frac{I_{\text{max}} \cdot S^n}{K^{n}_{\text{max}} + S^n} \]  

(Eq. 1)

Presteady-state currents were integrated with time (see legend to Fig. 5) and charge movements (Q) fitted to the Boltzman relation (Equation 2) for which  

\[ Q = \frac{Q_{\text{max}} - Q_{\text{up}}}{1 + \exp[(V_m - V_{0.5})/\Delta V]} \]  

(Eq. 2)

RESULTS

Steady-state Evoked Currents—In Na⁺ medium at pH 5.0, Gly-Sar evoked concentration-dependent inward currents (I GS ) which bore a nonlinear dependence upon membrane potential (V_m) in oocytes injected with hPEPT1 cRNA (Fig. 1). At positive V_m (−50 mV), the evoked currents asymptomatically approached zero current (Fig. 1A); the diminishing slope of the I/V_m relationship at +50 mV suggested no reversal (i.e. intracellular H⁺ and substrate concentrations were negligible). Beginning at +50 mV there was a region of marked voltage dependence which extended to −70 mV for 1 mW Gly-Sar and to −130 mV for 5 mW Gly-Sar, with a region of voltage-independence (or negative slope with V_m; see “Discussion”) at more hyperpolarized V_m. The evoked currents saturated with hyperpolarization for subsaturating (≤5 mW) Gly-Sar concentrations, but for 20 mW did not saturate with V_m within the V_m range tested. The I/V_m relationships for 20 mW (saturating) Gly-Sar for rabbit and human PEPT1 were virtually superimposable (Fig. 1B). Gly-Sar-evoked currents were unchanged in Na⁺-free medium at pH 5.0 (data not shown) but were significantly attenuated in Na⁺ medium at pH 7.5 (e.g. see Fig. 2A), suggesting that the Gly-Sar-evoked current is carried by protons.

At a fixed Gly-Sar concentration (1 mW), I GS increased in magnitude as a hyperbolic function of extracellular proton concentration ([H⁺] o), with Hill coefficient (n H) for H⁺ ~ 1; representative data at −110 mV is presented (Fig. 2A). At hyperpolarizing V_m (−90 to −130 mV), the apparent affinity constant of hPEPT1 for H⁺ ([H⁺] 0.5 ) was largely voltage-insensitive and approached an asymptote of ~50 mW at hyperpolarizing limits (Fig. 2B). From −70 mV, K GS 0.5 increased markedly with depolarization to ~1 μM at −30 mV. Switching the superfusing Na⁺ medium from pH 7.5 to 5.5 resulted in a shift in baseline current of 15 ± 1 nA (mean ± S.E., n = 6 oocytes), no greater than that observed for vehicle-injected oocytes, 12 ± 3 nA (n = 4).

The apparent affinity constant for Gly-Sar (K GS 0.5 ) at pH 7.0 increased from 1.2 to 2.5 mW as the cell was depolarized from −150 to −50 mV (Fig. 3A). Such a decrease in substrate affinity with depolarization is commonly observed for other transporters, e.g. SGLT1 (7) and SGLT2 (10), and since [H⁺] o = K GS 0.5 (see Fig. 2A) it is probable that the K GS 0.5/V_m relationship at pH 7.0 is a reflection of the voltage dependence of K GS 0.5 . However, the voltage dependence of K GS at pH 5.0 was very different; K GS at −150 mV was ~4 mW and fell with depolarization, reaching a minimum of 0.3 mW at +30 mV. The relationship of K GS 0.5 to V_m at pH 6.0 and 5.5 (data not shown) bore intermediate resemblance to that at pH 5.0 and at 7.0 (Fig. 3A). The Hill coefficient for Gly-Sar was 1 at each V_m tested. The relationship of the
Fig. 2. Proton-dependent activation of oligopeptide transport via hPEPT1. A, representative data (at $V_m = -110$ mV) showing the dependence of the 1 mM Gly-Sar-evoked current ($I_{GS}$) upon external proton concentration ($[H^+]_o$) determined at pH 7.5, 6.5, 6.0, 5.5, and 5.0 in a single hPEPT1 cRNA-injected oocyte. Data were fitted to Equation 1. The Hill coefficient ($n_H$) calculated using the data for $-110$ mV was $1.1 \pm 0.1$, an apparent stoichiometry of $1H:1$ Gly-Sar; at each voltage, $n_H$ approached 1.

Fig. 3. Kinetics of the Gly-Sar-evoked currents for hPEPT1. A, voltage dependence of the apparent affinity constant ($K_{0.5}^{GS}$) for Gly-Sar. The kinetic parameters of Gly-Sar transport were determined by measuring the currents evoked by Gly-Sar at 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 mM, each at pH 7.0, 6.0, and 5.0, in a single cRNA-injected oocyte, and derived according to Equation 1.

B: voltage dependence of the maximum current evoked by Gly-Sar, $I_{GS}^{max}$. Maximal Gly-Sar-evoked current ($I_{GS}^{max}$) to $V_m$ at pH 5.0 (Fig. 3B) followed the shape of the $I/V_m$ relation for 20 mM Gly-Sar (Fig. 1). At $-150$ mV, relative to $K_{GS}^{0.5}$, $I_{GS}^{max}$ was essentially independent of pH between pH 5.0 and 7.0 (Fig. 3B); however, with depolarization, $I_{GS}^{max}$ was significantly attenuated at diminishing $[H^+]_o$. The $I/V_m$ relationship was shifted left with increasing pH (at pH 7.0, the 1 mM Gly-Sar-evoked currents approached a zero current asymptote by $\approx +10$ mV), but the slopes of the $I/V_m$ curves were similar.

The $I/V_m$ relationships for 1 mM Gly-Sar were explored in the pH range 5.0–7.5 (Fig. 4A): at pH 7.5 the Gly-Sar-evoked currents were markedly voltage-dependent between $-150$ and $-50$ mV, and by $-30$ mV diminished close to zero. At subhyperpolarized potentials ($V_m \approx -90$ mV) the 1 mM Gly-Sar-evoked current increased with decreasing pH from 7.5 to 5.0, but at $-150$ mV the evoked current was greater at pH 6.0 than at pH 5.0, as a consequence of the increased $K_{GS}^{0.5}$ observed with hyperpolarization at pH 5.0 (see Fig. 3A). Selected data from Fig. 3 were re-plotted as a function of $[H^+]_o$ (Fig. 4, B and C). At 0.1 mM H$^+$, $K_{GS}^{0.5}$ was lower at $-150$ mV than at $-50$ mV; however, at $-150$ mV, increasing $[H^+]_o$ resulted in an increase in $K_{GS}^{0.5}$ whereas, at $-50$ mV, $K_{GS}^{0.5}$ fell by 72% between 0.1 and 1.0 mM H$^+$ and remained constant as $[H^+]_o$ was increased further to 10 mM. At $-150$ mV, the $I_{GS}^{max}$ fell only modestly (28%) as $[H^+]_o$ was reduced 100-fold from 10 to 0.1 mM (Fig. 4C). For $-50$ mV, although the drop in $I_{GS}^{max}$ was proportionately more marked than at $-150$ mV, $I_{GS}^{max}$ only fell significantly once $[H^+]_o$ was well below the $K_{0.5}^{H^+}$ ($\sim 0.4 \mu M$, for 1 mM Gly-Sar).

Pre-steady-state Currents—Following step changes in $V_m$ in oocytes expressing hPEPT1 we observed pre-steady-state currents (i.e. transient charge movements, e.g. see compensated record at pH 5.0, Fig. 5A) which were abolished by the addition of Gly-Sar. We investigated the relaxation kinetics for these transient currents: the transient currents relaxed with time constants ($\tau_{on}$, “on” currents) in the range 2–10 ms, and the relationship of $\tau_{on}$ to $V_m$ appeared bell-shaped (Fig. 5B). By varying pH between 5.0 and 6.5, we observed that the peak $I_{GS}$ ($i.e. \tau_{on}$) increased with increasing $[H^+]_o$ (Fig. 6A), and the $V_m$ at which $\tau_{on}$ was maximal (i.e. $V_m^{max}$) moved in the hyperpolarizing direction with increasing pH (Figs. 5B and 6B). The relaxation time constants were different for “on” and “off” currents (Fig. 5A): for off currents, $\tau$ varied neither with $V_m$ nor pH ($\tau_{off} \approx 6$–7 ms, Fig. 5B). However, the charge movements were of the same magnitude for each (not shown). Charge movements were averaged (on and “off”) and fitted to the Boltzmann relation (Equation 2, Fig. 5C): the maximal charge transloc-
tion (Q_max) apparently dropped 23% as [H⁺]o was reduced from 10 to 0.32 mM (Fig. 6D). The V_m for 50% charge translocation (V_0.5) was linearly dependent on pH: in two separate oocytes, the slope of the V_0.5/pH relation was 1.75 mV (Fig. 6C) and 1.84 mV (not shown) per 10-fold increase in [H⁺]o. The pH dependence of V_0.5 was quantitatively similar to that of V_{max} (Fig. 6B) at ~80 mV per 10-fold change in [H⁺]o. Furthermore, at any given pH, V_{max} and V_0.5 coincided within 15 mV (Fig. 6B). The apparent valence of the movable charge (z) was ~1 and was reduced only slightly with increasing [H⁺]o (Fig. 6E).

DISCUSSION

A significant fraction of the dietary amino nitrogen is absorbed as intact oligopeptides rather than free amino acids (11). Ganapathy and Leibach (2), and more recently Meredith and Boyd (1) have reviewed H^+/ oligopeptide transport in the epithelia of kidney, intestine, lung, and placenta, and the blood-brain barrier, in which dipeptides and tripeptides are accepted as substrates. Oligopeptide transport activity is evidently served by more than one transport protein both in rabbit intestine (12) and kidney (13), and a second H^+/oligopeptide transporter (hPEPT2) from human kidney was recently cloned (14) and characterized (15). Kinetic analysis demonstrated that Gly-Sar and Gly-Pro share a common pH-gradient-dependent carrier system in rabbit intestinal brush-border membrane vesicles (16) but a multiplicity of transport systems was suggested by the additive effects of several other oligopeptides expressed in oocytes. All data described are for a single hPEPT1 cRNA-injected oocyte at 20–22°C in the absence of substrate, and were replicated in a second oocyte (not shown) with Q_{max} of up to 12 nC. A, representative carrier-mediated transient currents at pH 5.0: these were obtained from the total currents by subtraction of the currents due to membrane capacitance (with a decay constant of ~0.7 ms) and the steady-state currents, using the fitted method previously described (9). Currents (filtered at 100 Hz for display) are shown from 3 ms after the voltage steps indicated in the top panel. B, kinetics of transient current relaxation (for on currents), were described by a single time constant (t_{on}, filled symbols) between 3 ms and upon reaching steady-state at pH 5.0, 5.5, 6.0, and 6.5 (solid lines are fitted Gaussian relations). The time constants for the off currents (t_{off}) did not vary with either V_m or pH. C, charge-voltage relationships for hPEPT1 at pH 6.5, 6.0, 5.5, and 5.0 obtained using the fitted method (9) (filled symbols); at each pH, the data were fitted to the Boltzmann equation (Equation 2) (solid lines). Q/V_m data were normalized such that the depolarizing limits of Q were equalized for each pH value.
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Fig. 6. Presteady-state kinetic parameters for hPEPT1 described as a function of extracellular protons. Presteady-state kinetic parameters were derived from data in Fig. 5. A, effect of external proton concentration upon the maximal τw value, τwmax, B, the voltage at which τw is maximal, Vτw; the slope of the linear regression (solid line, r = −0.93) is −83 mV per pH unit (10-fold change in [H⁺]). C, the effect of [H⁺o] upon Vτw, the potential for 50% charge translocation; the linear regression (solid line, r = −0.99) indicated a shift in Vτw of +75 mV per 10-fold increase in [H⁺o]. (In a second cRNA-injected oocyte, Vτw shifted +84 mV per 10-fold increase in [H⁺]; r = −0.99.) D, effect of pH upon the maximal charge translocation, Qmax; E, the effect of [H⁺o] upon the apparent valence, z, of the movable charge (related to the slopes of the Q/Vm relationships in Fig. 5C).

(12). In this latter study, the authors used a potential-sensitive fluorescent dye to demonstrate that oligopeptide transport was electrogenic; furthermore, imposing a valinomycin-induced K⁺diffusion potential could almost double Gly-Pro transport (17).

Expression of the cloned rabbit intestinal H⁺/oligopeptide cotransporter rPEPT1 in Xenopus oocytes (5) revealed that Gly-Sar transport (K₂₅₀ ≈ 1.9 mM) was electrogenic and independent of Na⁺, Cl⁻, and K⁺, and that rPEPT1 transported a broad range of dipeptides, tripeptides, and β-lactam antibiotics with K₀.₅ ≈ 0.1–5 mM. Surprisingly rPEPT1 displayed high apparent affinity for the anionic dipeptide alanyl-aspartate (K₀.₅ ≈ 140 μM), whereas the rabbit intestinal brush-border membrane vesicles neutral dipeptides, or those bearing a single positive charge, were generally favored (18). The human homologue (4) exhibited higher affinity for Gly-Sar than rPEPT1 but similar substrate scope: Gly-Sar transport in hPEPT1-transfected HeLa cells (K₀.₅ = 0.3 mM, pH 6.0) was inhibited by several dipeptides, tripeptides, and β-lactam antibiotics, but not by free amino acids (4).

Gly-Sar transport via rPEPT1 proceeded with a pH optimum of 5.5, and intracellular pH recording yielded the first convincing evidence that oligopeptide uptake was coupled to a H⁺ flux (5). Boll et al. (6) confirmed that uptake of the antibiotic cefadroxil was also H⁺-coupled, but with a pH optimum of 6.5. The Hill coefficient for H⁺ obtained for Gly-Sar uptake (5) was 1, consistent with Hill plots for H⁺ activation of Gly-Gln uptake mediated by both the high and low affinity systems in rabbit renal brush-border membrane vesicles (13).

Whereas Fei et al. (5) investigated the voltage dependence of oligopeptide transport by rPEPT1 using two-microelectrode voltage-clamp and concluded that Gly-Sar transport was almost completely independent of voltage (in the range −150 to +50 mV), Boll et al. (6) found transport of both Gly-Sar and cefadroxil to be voltage-dependent, with the Gly-Sar-evoked current saturating with hyperpolarization (see below).

The present study was designed to elucidate the molecular mechanisms by which hPEPT1 transports small peptides. By measuring evoked currents in Xenopus oocytes expressing the human H⁺/oligopeptide transporter hPEPT1, we have demonstrated that Gly-Sar transport via hPEPT1 is electrogenic and coupled to an inward H⁺ current. For both the human and rabbit PEPT1 transporters we observed that evoked currents for saturating Gly-Sar concentrations (~20 mM) were voltage-dependent over the entire Vm range tested (~150 to +50 mV). For rPEPT1 it is unclear why there were differences between this study and that of Fei et al. (5); certainly the poor expression of rPEPT1 obtained by Fei et al. (<100 nA), compared with up to 2,000 nA in this study (Fig. 1B), made it difficult to detect any changes in the evoked current with Vm. However, the data shown in Fig. 4F of Ref. 5 can be alternatively explained: the 10 mM (subsaturating) Gly-Sar-evoked current was Vm-dependent between +50 mV and −110 mV, with a region of negative dependence upon Vm below −110 mV, similar to that observed for saturating Gly-Sar concentrations in hPEPT1 (Fig. 1A). In contrast to that obtained by Boll et al. (6) for 10 mM Gly-Sar, the 1/Vm relationship for rPEPT1 for 20 mM Gly-Sar showed no evidence of saturating with Vm by −150 mV (Fig. 1B), but this is consistent with the concentration-dependent shift in the Vm at which the evoked currents saturate for hPEPT1 (Fig. 1A, see below).

We found that H⁺/Gly-Sar cotransport in oocytes expressing hPEPT1 obeyed Michaelis-Menten-type kinetics. The apparent affinity constant for Gly-Sar (K₂₅₀) was ~0.7 mM at Vm = −50 mV (in the pH range 5.0–6.0), of the same order as that obtained from radiotracer fluxes for hPEPT1 expressed in non-voltage-clamped HeLa cells (0.3 mM at pH 6.0) (4), and slightly lower than the K₂₅₀ reported for rPEPT1 (1.9 mM at −60 mV, pH 5.5) (5). At each Vm, Hill coefficients were ~1 for H⁺ activation of the Gly-Sar-evoked current (Fig. 2A), and also for Gly-Sar activation, suggesting 1H⁺:1 Gly-Sar transport stoichiometry.

Following step changes in Vm, in the presence of H⁺, but in the absence of Gly-Sar, we observed presteady-state (transient) currents similar to those observed for several other cloned transport proteins expressed in oocytes, e.g. the intestinal Na⁺/glucose cotransporter SGLT1 (9) (see Table I). These charge movements for hPEPT1 were fitted to the Boltzmann relation (Equation 2, Fig. 5C). We observed a modest dependence of maximal charge transfer (Qmax) upon [H⁺], (Fig. 6D) whereas in contrast the dependence of Vτw (the membrane potential at 50% Qmax) upon [H⁺] was very dramatic: Vτw was linearly dependent upon pH with a slope of ~80 mV per 10-fold increase in [H⁺] (Figs. 5C and 6C). This represented a larger shift in Vτw due to changes in activator concentration than for human SGLT1, but it was less marked than the Na⁺-dependent shifts in Vτw observed for the low affinity SGLT2, the Na⁺/glutamate cotransporter (EAAT2) and the Na⁺/Cl⁻/GABA cotransporter (GAT1). The transient currents associated with hPEPT1 relaxed with time constants of 2–10 ms, broadly com-
parable to those for SGLT1, SGLT2, SMIT (canine Na+/inositol cotransporter), the plant H+/inositol cotransporter (STP1) and EAAT2; however, current transients for GAT1 decay much more slowly (Table I). \( \tau \) appeared to follow a bell-shaped relationship to membrane potential (Fig. 5B): lowering \([\text{H}^+]_o\) also reduced the extent to which \( \tau \) varied with \( V_m \). Increasing pH from 5.0 to 6.0 reduced \( \tau \) from \( \sim 10 \) to \( \sim 8 \) ms; and the membrane potential at which \( \tau \) was obtained also appeared to shift \( \sim 80 \) mV for a 10-fold increase in \([\text{H}^+]_o\), consistent with the slope of \( V_o/pH \) (Fig. 6).

The partial reactions involved in the presteady-state currents are illustrated in Fig. 7B; each reaction step was treated as a function of the first order \((k_{51}, k_{16}\) and \(k_{50}\)) or pseudo-first order \((k_{12})\) rate constants. Modelling of the partial reaction followed the principles described for rabbit and human SGLT1 (9, 19), modified for one ion-binding site. According to this model presteady-state currents are due to (i) binding/dissociation of \( \text{H}^+ \) to/from the cotransporter and (ii) a conformational change of the unloaded carrier between the external and internal membrane interfaces. The phenomenological constant \( \alpha \) describes the fraction of the membrane field between extracellular \( \text{H}^+ \) and the \( \text{H}^+\)-binding site at the external face, \( \alpha \) is its internal equivalent, and \( \delta \) is the fraction of the membrane field sensed by the empty binding site on the carrier during translocation; microscopic reversibility requires that \( \alpha + \alpha^* + \delta = 1 \) (20). Simulations indicated that this model can quantitatively account for the \( Q/V_m \) and \( \tau/V_m \) relationships observed for hPEPT1 at pH 5.5 (Fig. 7, C and D, see legend for the values of each constant); the model predicted \( \alpha \) \( \sim 0 \), i.e. \( \text{H}^+ \) binding at the internal face is essentially independent of voltage, in common with intracellular Na\(^+\) binding for SGLT1 (9, 19). The model also qualitatively described (not shown) (i) the reduction in \( Q/V_m \) as pH rises (a reflection of the relative contribution of \( \text{H}^+\)-binding/dissociation to the overall charge transfer) together with the shift in \( V_{0.5} \) to more hyperpolarized \( V_m \); and (ii) the reduction in \( \tau/V_m \) from 5.0 to 6.5 together with the pH-dependent shift in \( V_{0.5} \) which parallels that of \( V_m \).

\([\text{H}^+]_o\) modulates charge transfer across the membrane over a broad voltage range (Figs. 5C and 6, B and C). The apparent valence of the movable charge on the carrier (\( z \), which may represent the aggregate of more than one charge transfer step) was around \( 1 \) at each \( \text{pH} \) as for other transporters except EAAT2 (Table I), and the \( Q/V_m \) relationship extends over a wide voltage range at any given \([\text{H}^+]_o\). That is, the extent of the charge transfer (and subsequently therefore also the magnitude of the steady-state current) is finely regulated according to \( V_m \). The value of \( z \) fell by one-third over the \([\text{H}^+]_o \) range 0.32-10 \( \mu \text{M} \) (Fig. 6E), so that carrier orientation was biased over a broader \( V_m \) range at high \([\text{H}^+]_o\) than at low \([\text{H}^+]_o\), whereas for human SGLT1 \( z \) was independent of \([\text{Na}^+]_o\) (9).

The ratio of \( I_{\text{max}} \) at \( -150 \) mV to \( Q_{\text{max}} \) at saturating \([\text{H}^+]_o\) is an index of the turnover rate of the transporter (9); in two oocytes the turnover rates were 97 and 96 s\(^{-1}\); however, these are underestimated since the carrier was not voltage-saturated at \(-150 \) mV (see Fig. 1 and Fig. 3B). The turnover rates for hPEPT1 and rPEPT1 are 2-10-fold greater than those determined for other transporters (see Table I). Using the relation \( Q_{\text{max}} = C_r z e \), where \( C_r \) represents the carrier density, and \( e \) the elemental charge, at \( \text{pH} 5.0 \) (\( Q_{\text{max}} = 12 \text{nC} \)), we estimated hPEPT1 carrier density to be \( \sim 10^5 \) per oocyte, within an order of magnitude of the density values obtained for other transporters.

Presteady-state data indicated that \( \text{H}^+ \) can bind to the empty carrier in the absence of oligopeptide. The steady-state data presented indicated that \( \text{H}^+ \) and oligopeptide are translocated simultaneously in the same reaction step. This is supported by the observation that, at least at \( V_m \) more positive than \( -70 \) mV, the affinity of the carrier for substrate (Gly-Sar) deteriorated at diminishing \([\text{H}^+]_o\), e.g. at \( -50 \) mV, \( K_{50} \) rose sharply at \([\text{H}^+]_o \leq 1 \mu \text{M} \) (Fig. 4B) (21). The transporter prefers to bind \( \text{H}^+ \) and substrate in an orderly fashion, \( \text{H}^+ \) first. This conclusion is based upon the observation that the apparent maximal transport rate \( (I_{\text{max}}^{\text{Gly-Sar}}) \) was barely attenuated when \([\text{H}^+]_o\) was reduced 10-fold from 10 to 1 \( \mu \text{M} \) (Fig. 4C). Only once \([\text{H}^+]_o\) was reduced 100-fold (to well below the \( K_{50} \)) did \( I_{\text{max}}^{\text{Gly-Sar}} \) fall markedly, and even then the reduction in \( I_{\text{max}}^{\text{Gly-Sar}} \) was less than would be expected for a system in which activator and substrate binding were random (21, 22).

At \( \text{pH} 7.5 \), the current evoked by 1 \( \mu \text{M} \) Gly-Sar was \( \sim 30\% \) that at \( \text{pH} 5.5 \) (Fig. 2A) whereas [\(^{14}\text{C}\)Gly-Sar uptake (at 30 \( \mu \text{M} \) Gly-Sar, \( \text{pH} 7.5 \)) was as much as 54\% that at \( \text{pH} 5.5 \) (4). The reason for the discrepancy is not clear, but this may indicate that there is an appreciable \( \text{H}^+\)-uncoupled flux of Gly-Sar at \( \text{pH} 7.5 \). Thus we propose a model (Fig. 7A) in which the preferred route is for \( \text{H}^+ \) to bind first (states 1→2) then Gly-Sar (states 2→3), and for \( \text{H}^+ \) and Gly-Sar to be translocated simultaneously (states 3→4), but not excluding the possibility that a \( \text{H}^+\)-uncoupled Gly-Sar flux may proceed (states 1→2a→5a) at high \( \text{pH} \). An uncoupled \( \text{H}^+ \) flux (states 2→5) is unlikely since switching the superfusing \( \text{Na}^+ \) medium from \( \text{pH} 7.5 \) to 5.5 (in the absence of peptide) resulted in a shift in baseline current in oocytes expressing hPEPT1 no greater than that observed for vehicle-injected oocytes. That \( K_{50} \) increased markedly with depolarization (Fig. 2B) indicated that, at least within the
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A

\[
\begin{array}{cccccc}
\text{OUT} & \text{IN} \\
1 \rightarrow [C]^\prime & [S]^\prime & 6 \rightarrow [C]^\prime \\
2 \rightarrow [CS]^\prime & [S]^\prime & 5 \rightarrow [CS]^\prime \\
3 \rightarrow [CHS]^\prime & [S]^\prime & 4 \rightarrow [CHS]^\prime \\
[HK]^\prime & [S]^\prime & [HK]^\prime \\
2a \rightarrow [CS]^\prime & [H]^\prime & 2 \rightarrow [CH]^\prime \\
5a \rightarrow [CS]^\prime & [H]^\prime & 5 \rightarrow [CH]^\prime \\
3 \rightarrow [CHS]^\prime & [H]^\prime & 4 \rightarrow [CHS]^\prime
\end{array}
\]

\[k_{\text{exp}}(0.5\mu\text{M}) \text{ or } k_{\text{exp}}(0.5\mu\text{M})\]

B

\[k_{\text{exp}}(0.5\mu\text{M}) \text{ or } k_{\text{exp}}(0.5\mu\text{M})\]

C

\[\tau_{\text{in}}(\text{ms})\]

D

\[Q/Q_{\text{max}}\]

Fig. 7. Kinetic model for the operation of the human H⁺/oligopeptide cotransporter hPEPT1. A, an eight-state model of hPEPT1 in which the empty carrier is negatively charged (the apparent valence of the movable charge is −1, and the Hill coefficient is 1). Each carrier state is identified by a number; carrier states at the external face of the membrane are further identified by a prime and, at the internal face, by double prime. Essentially the model is similar to that proposed for SGLT1 (19) except that two additional states (2a and 5a) are added since Gly-Sar may bind in the absence of protons and an appreciable “internal leak” may proceed uncoupled to protons; this substrate leak pathway (states 2a–5a) is shown as a dotted line to represent the assumption that translocation of the fully loaded carrier (states 3–4) is the favored pathway. We do not consider a H⁺ leak pathway (states 2–5, see text for justification). B, presteady-state currents observed in the presence of external protons can be accounted for by a three-state (states 6, 1, and 2) partial reaction since transient charge transfers are abolished by Gly-Sar. Membrane potential affects both the translocation of the empty carrier (states 6–1) and proton binding with the carrier (states 1–2). The partial reaction scheme is described by four membrane potential-dependent rate constants \(k_{\text{on}}, k_{\text{off}}, k_{\text{12}}, k_{\text{11}}\) (9, 19): a rate constant \(k_{\text{on}}\) (for a reaction step \(x \rightarrow y\)) is defined by its potential-independent value \(k_{\text{on}}^0\), \(V_{\text{m}}\), and ligand (H⁺) concentration, as well as the coefficients \(\alpha, \alpha', \beta\), and \(\delta\) which describe the fraction of the electric field sensed by the H⁺ binding to its external site \(\alpha\) or internal site \(\alpha'\) and by the empty ion binding site on the carrier during membrane translocation \(\delta\), where \(\alpha + \alpha' + \beta + \delta = 1\) (20); \(\mu\) is the electrical potential \(FV_{\text{m}}/\text{RT}\). C and D, model prediction of charge transfer associated with hPEPT1, based on simulation of the three-state model in voltage range −70 mV to −30 mV, H⁺ binding is voltage-dependent. According to our model, the modest dependence of \(Q_{\text{max}}\) upon \(\mathrm{H}^+\) (Fig. 6D) supports the conclusion that H⁺ binding is voltage-dependent, i.e., “ion well” effect (21), but that reorientation of the empty, charged carrier within the membrane field accounts for most of the charge movements observed.

The interpretation of the data is complicated under conditions of large hyperpolarizing potentials and high \(\mathrm{H}^+\), by a nonspecific inhibitory effect of pH upon substrate binding (illustrated best by Fig. 4A). This may be due to chemical perturbations of the protein structure at low pH since (i) hyperpolarization and high \(\mathrm{H}^+\) will together maximize the number of empty carriers exposed to the extracellular milieu, and (ii) the manifestation of this pH effect is countered by increasing the [Gly-Sar] (see Fig. 1A).

The \(V_{\text{m}}\), at which the I/\(V_{\text{m}}\) relationships saturated with \(V_{\text{m}}\), moved in the hyperpolarizing direction as [Gly-Sar] was increased (Fig. 1A) in contrast to SGLT1 (7) and SGLT22 for which saturation with \(V_{\text{m}}\) is reached at more depolarized \(V_{\text{m}}\) with increasing sugar at fixed [Na⁺] (20). This feature of hPEPT1 is presumably a direct result of the reduced affinity of the carrier for Gly-Sar at low pH and hyperpolarization. Since this effect is ameliorated by increasing substrate concentration, it may be equally noticeable at higher pH when using a substrate of lower apparent affinity than Gly-Sar.

hPEPT1 shares general features in common with the Na⁺-coupled transporters: steady-state and presteady-state kinetics for hPEPT1 were reminiscent of those for SGLT1 (7, 9), SGLT2 (10), and SMIT (23). The biophysical parameters determined for hPEPT1 (and rPEPT1), and the nature of their voltage dependence, were similar to the cloned Na⁺-coupled transporters (Table I). This observation (i) argues against the notion that protons behave inherently differently to Na⁺ in coupled transporters, suggesting instead that the mechanical organization of hPEPT1 and the mechanism of cation activation are similar to Na⁺-coupled transporters; and (ii) opposes the idea that H⁺-coupled transporters in mammals represent a phylogenically “primitive” class of transport proteins (24). H⁺ is equally effective as Na⁺ in driving α-methyl-D-glucopyranoside transport via SGLT1 (25).

In summary, we have shown that glycyrl-sarcosine evoked voltage-dependent and H⁺-dependent currents in oocytes expressing the human H⁺/oligopeptide cotransporter hPEPT1. At resting membrane potential (−50 mV), H⁺ behaved as an essential activator of oligopeptide transport and the apparent affinity for Gly-Sar was maximal when pH was close to that measured (pH 6.0) in human proximal jejunal in situ (3). Under these conditions, \(\mathrm{H}^+\) (1 μM) was ~2.5 times greater than the measured half-maximal \(\mathrm{H}^+\) (5). Excluding a nonspecific effect of low pH at hyperpolarized potentials, our data are consistent with an ordered, simultaneous transport model in which H⁺ binds first. Model simulation provided single-step rate constants which can account for the presteady-state

\[B \text{ (see text) for which } \alpha = 0.27, \alpha' = 0, \beta = 0.73, k_{\text{on}}^0 = 82 \text{ s}^{-1}, k_{\text{off}} = 310 \text{ s}^{-1}, k_{\text{12}} = 1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}, k_{\text{11}} = 550 \text{ s}^{-1}, \text{ and } [\mathrm{H}^+] = 3.16 \times 10^{-6} \text{ M (pH 5.5). The model predictions (solid lines) of } \tau_{\text{in}} \text{ and } Q \text{ for pH 5.5, obtained as described previously (9, 19), were compared with the actual data (A) extracted from Fig. 5, B–C. C, } \tau_{\text{in}} \text{ as a function of } V_{\text{m}}, \text{ the model also predicts a second much faster time constant } \tau_{\text{on}}, \text{ not shown, refer to Ref. 9 which exceeds the resolution of the current technique. D, Q as a function of } V_{\text{m}} \text{ normalized by } Q_{\text{max}}, \text{ the maximal charge at extreme depolarizing potentials.}

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charge movements observed for hPEPT1 in the absence of Gly-Sar, and attributed to reorientation of the empty carrier in the membrane and in part to H⁺ binding/dissociation. Investigating the kinetic characteristics of the products of site-directed mutagenesis in hPEPT1, and those of a second H⁺/oligopeptide cotransporter, hPEPT2 (14), ought to provide insights into structure-function relationships for this family of transport proteins.

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